ICE NUCLEATING AGENTS INVOLVED IN FREEZING OF PLANT TISSUES

By

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Cold hardiness and equilibrium freezing curves were determined for acclimated citrus leaves varying in hardiness. Freeze avoidance capability of tender plants was examined as related to the presence of ice nucleation active bacteria. Freezing dynamics as well as methods of preventing bacterial ice nucleation were explored.

Citrus leaves varied in cold hardiness from -4° (Citrus limon L.) to -11°C (C. unshiu Marc.). Liquid water content (g H_2O/g dry wt) of unfrozen samples as well as melting point depression (from solutes) were not significantly different among species. Equilibrium freezing curves for lime, grapefruit, orange, and mandarin leaves were very similar. The tissues deviated from ideal freezing behavior. Reduced ice formation could be accounted for by the formation of negative pressure potential during freezing. Expressed sap, tissue prefrozen in liquid nitrogen, and thawing curves exhibited freezing behavior closer to ideal than intact tissue.

Tender plants are killed when frozen. Presence of the epiphytic bacteria, Pseudomonas syringae and Erwinia herbicola, caused plants to freeze at higher temperatures. A threshold inoculum concentration of about 10^5 cells/ml was necessary for high temperature ice nucleation. Ice nucleation efficiency decreased with time (unless plants were maintained at high relative humidity) but higher percentages of inoculated plants froze compared to control plants even after 17 days.

Ice nucleation is a dynamic property of P. syringae. Cells grown at 22° were much more efficient ice nucleators than when grown at 30°C. The subsequent storage temperature (of static cultures) markedly affected the efficiency of ice nucleation. The temperature at which 20 µl drops froze was 5°C warmer for bacteria stored at 5°C (compared with storage at 22°C). Changes in effectiveness were reversible. The viable bacterial concentration remained constant, indicating a switching mechanism by living cells from nucleation active to nucleation inactive.

The use of competitive bacteria did not significantly reduce frost damage to tomato plants. Spectinomycin reduced freezing percentages while streptomycin was effective only when combined with salts or added to the bacterial suspension before inoculation. Epiphytic bacterial populations are reduced in number by ultraviolet-β radiation.

INTRODUCTION

Plants exhibit a broad range of resistance to freezing temperatures. Tender plants are killed when frozen while some hardy types cold acclimate to tolerate liquid nitrogen temperature (-196°C) (67, 76,78). There are two basic survival mechanisms. Plants may either tolerate or avoid freezing stress. Frost-tolerant plants can survive extracellular freezing while frost-susceptible plants must avoid freezing to survive (98). Variability in plant response to freezing stress results in frost killing temperatures ranging from a few degrees below zero (C) to below those encountered anywhere on earth.

Hardy plants cold acclimate to tolerate freezing temperatures. Environmental cues as well as endogenous rhythms trigger biochemical and physiological changes in the plant. These changes enable the plant to tolerate extracellular freezing. Differences in hardiness between species is best explained by differences in the amount of frozen water that is tolerated in the tissue. Although a universal mechanism of damage is not known, it is generally accepted that the plasma membrane is the primary site of damage.

Most citrus plants have the capacity to cold acclimate. The most cold-hardy citrus acclimate to about -10°C. Sweet oranges generally tolerate about -6°C while lemons and limes are very cold tender. Citrus species acclimate only a few degrees and therefore are plagued by intermittent freeze damage.

Frost-susceptible plants do not cold acclimate and therefore do not tolerate any ice formation. These tender plants survive freezing temperatures by supercooling. This is possible only in the absence of ice-nucleating agents. The most efficient natural ice nucleators are bacteria of the Pseudomonas and Erwinia genera. These bacteria cause tender plants to freeze and be killed at a much higher temperature than if the bacteria are not present. Since these bacteria are reported to be ubiquitous they are considered primary factors in inducing frost damage to tender plants (49).

The primary objectives of this research were twofold. First, the freezing process in citrus leaves was examined to determine whether differences in hardiness could be explained by differences in freezing behavior. Also, the effects of ice nucleation active bacteria on supercooling of tender plants were examined and possible control methods were explored.

Viability testing for citrus leaves was developed and the acclimation-deacclimation sequence is described in Appendix 1. Field surveys of epiphytic bacterial populations were carried out and the results are summarized in Appendix 2.

REVIEW OF LITERATURE

Freezing temperatures limit production of many crops (1,61, 92,97). Fruit industries in the United States lose more money to frost damage than from insects, diseases, rodents, and weeds combined (45). Florida experiences severe freezes with an average interval between freezes of 10 years (13), resulting in a $500 million loss to the citrus industry alone in 1962 (115). Freeze damage in 1977 claimed 30-35% of the Florida citrus crop (109) while tree damage was even greater in 1981 (110). Frost hardiness research has an enormous potential to reduce plant and crop losses.

Plant survival may be the result of tolerance or avoidance of frost stress (47). Frost tolerant plants can survive extracellular freezing while frost-susceptible plants must avoid freezing to survive (92). Death of tender plants results from spring and fall frosts while hardy plants can be killed by light frosts when not acclimated as well as by midwinter minima.

Frost-tolerant plants acclimate to low temperature in response to environmental cues as well as endogenous rhythms (84,93). Acclimation research has focused on red-osier dogwood (Cornus stolonifera Michx.). Dogwood can survive liquid nitrogen temperature when acclimated but is killed at slightly below 0°C when deacclimated (6, 22). The first stage of acclimation (-3 to -20°C) is phytochrome

mediated, requiring short days and high temperatures (35,63,77). A hardiness promoter, most likely ABA (abscissic acid), is produced in leaves in short days and translocated via the phloem (19,36,97). Leaves in long days are the photoreceptors for the production of a translocated hardiness inhibitor (38). Protoplasmic augmentation occurs during this stage of active metabolism. Protein, phospholipids, simple sugars, organic acids, total RNA, and energy charge (ATP/AMP+ADP) increase while starch, inorganic phosphorus and tissue hydration decrease (48). The change in water status is due to increased root resistance to water influx in addition to increased gas exchange by the leaves (62,68). Water stress can increase hardiness but is not cumulative with the effect of short days (11,12,68). Water stress apparently yields the same physiological end as short days (10). The second stage of acclimation requires frost. Extracellular freezing causes a severe dehydration stress as water leaves the protoplasm. The mechanism resulting in extreme hardiness (to -196°C) does not involve a translocated factor (18).

Growth cessation is a prerequisite to acclimation in dogwood (18). Deciduous plants typically undergo two phases of dormancy. A plant in quiescent dormancy will resume growth upon return to favorable conditions or as a result of cultural practices including pruning and nitrogen fertilization. A plant in rest will not grow. High temperatures during rest may result in decreased hardiness but a return to low temperature results in a return to maximum hardiness (70). The transition from rest to quiescence requires a characteristic time

interval in a specific temperature range. The particular requirement is a function of the genotype as well as the environmental conditions of the preceding season (88).

Citrus plants do not attain rest but remain quiescent in response to low temperatures (115). Low-temperature-induced dormancy is necessary for citrus to become cold hardy (118,119). Hardy species such as 'Satsuma' and 'Cleopatra' mandarin have a higher acclimation temperature threshold than less hardy species such as lemon and lime (113,118). Water-stress-induced dormancy has also been shown to increase hardiness in citrus plants (104,106).

The relative hardiness of citrus species (from most to least hardy) has been observed to be 'Satsuma' and 'Cleopatra' mandarin, sweet oranges, grapefruit, lemon and lime (100,111,113). The hardiness of these scion species is affected to a limited extent by the rootstock. Mandarin rootstocks were more hardy than citranges ('Savage' and hyrbids) (117) and 'Sour' orange (32) which impart more hardiness than lemons ('Iran' and 'Rough') and limes ('Rangpur' and 'Kalpi') (21). The relative hardiness of rootstocks has been reported to vary from the beginning to the end of the winter (108).

Light is necessary to provide photosynthate required for acclimation (100,114), but photosynthetic rates are substantially reduced upon attainment of hardiness (116). Changes in citrus metabolites during hardening are similar to the changes in dogwood (103). Sugars, mainly glucose, fructose, and sucrose, increase in leaves of hardened plants (40,83,107,114). Yelenosky found that

neither sugar nor proline accumulation was related to specific levels of hardiness (105).

Citrus stems are more hardy than leaves which, in turn, are hardier than fruit (33). The presence of functional leaves is necessary for hardening in the stems (101). Ice propagation was found to be slower in hardened stems (102).

Citrus cold hardiness has been estimated by the freezing temperature of leaves (24,37,39). In these studies the heat of fusion was sensed and considered indicative of the frost killing temperature. Young found that the leaf freezing temperature of citrus leaves was affected by the chamber temperature, hence the cooling rate (112). Decreased freezing points in the winter were observed in grapefruit leaves but they were not correlated with hardiness (119).

Tender plants do not cold acclimate. These plants survive temperatures lower than a few degrees below 0°C by avoiding equilibrium with the freezing stress. This is accomplished by supercooling of the tissue water. Water that is free of heterogeneous nucleators will supercool to about -38°C before homogeneous ice nucleation occurs (55, 99). Floral primordia of azalea (23), dogwood (79), and peach (71), and xylem ray parenchyma of hardwoods (72) deep supercool. Corn and wheat plants supercool to -10°C in laboratory tests (50,59), although field plants are not observed to supercool more than a few degrees (8). This paradox led Marcellos and Single on an unfruitful search for the agent(s) responsible for ice nucleation of wheat plants in the field (59,85).

Schnell and Vali (82) and Kaku (41) found efficient ice nucleation associated with poplar and Veronica persica leaves. However, it was not until Fresh (unpublished data) isolated a bacterium from alder leaves that it was established that the ice nucleation was a result of epiphytic bacteria. This bacterium was identified as Pseudomonas syringae by Maki and coworkers (56). Since then several studies have implicated epiphytic bacteria of the Pseudomonas and Erwinia genera as causal agents of heterogeneous ice nucleation at temperatures as warm as -2°C (3,49,51,56,69).

Erwinia herbicola, Pseudomonas syringae, and Pseudomonas fluorescens have been demonstrated to be active in ice nucleation (34, 50,57). Pseudomonas syringae, an ice nucleation active (INA) bacterium, is ubiquitous, infecting a broad spectrum of host plants (14). Pseudomonas syringae is found in residence on plants void of pathological symptoms (46,88) and in numbers sufficiently high to account for ice nucleation during winter months (49). Heterogeneous nucleators such as INA bacteria may play a major role in limiting tender crop production by initiating freezing at relatively warm temperatures.

CHAPTER I
CITRUS COLD HARDINESS

Introduction

Cold-acclimated citrus species exhibit a range of resistance to freezing. For example, cold-hardy 'Satsuma' mandarin tolerates temperatures below -10°C whereas foliage of cold-sensitive lemon and lime are killed at -4°C. Grapefruit and sweet orange are considered intermediate in hardiness (100,109,111).

All mechanisms of frost resistance must avoid intracellular freezing which is lethal in plant tissues (6,98). In citrus groves, the cooling rate is slow enough for water to freeze extracellularly in acclimated trees. Cells dehydrate and collapse as the amount of extracellular ice increases with decreasing temperature. Cell sap becomes concentrated as water leaves the protoplast forming extracellular ice, while intracellular freezing is avoided colligatively. That is, the intracellular freezing point is depressed due to an increase in solute concentration. The amount of ice formed (and the concomitant freezing stress) is largely determined by temperature, amount of freezable water, and the pressure, matric and osmotic potentials of the cell.

Increased frost resistance in cold-acclimated plants, as well as differences between species, have been associated with increased cell sap concentration (47). A high concentration of cell solutes requires a lower temperature to form the same amount of ice formed in a

more dilute solution of equal volume. Soluble sugars increase in lemon, lime, grapefruit, orange, and mandarin plants during winter months (40,83,114). However, sugar accumulation does not account for specific levels of cold hardiness in citrus (105,114). A cause-and-effect relationship between soluble sugar concentration and cold hardiness has not been observed in other plants. Concentrations of soluble sugars in winter wheat are correlated with total sugars (17) but there are instances where sugar concentration is inversely related to cold hardiness (27). Also, osmotic potential and hardiness level were not correlated in Solanum species (9).

The primary objective of this study was to examine freezing behavior as a possible basis for differences in cold hardiness in citrus species. A secondary objective was to establish the relationship between osmotic potential and plant water potential at freezing temperatures.

Materials and Methods

Terminal shoots of 'Lisbon' lemon (Citrus limon (L.) Burm. f.), 'Ruby Red' grapefruit (Citrus paradisi Macf.), 'Valencia' orange (Citrus sinensis (L.) Osbeck), and 'Satsuma' mandarin (Citrus unshiu Marc.) were collected from Polk County, Florida, on January 21, 1982, and packaged in plastic bags. The bags containing the citrus shoots were placed in a styrofoam cooler along with damp towels to prevent desiccation. The packaged leaves were flown to the Crop Development Centre of the University of Saskatchewan in Saskatoon, Saskatchewan,

where nuclear magnetic resonance (NMR) analyses were done from January 23 to February 1, 1982.

Nuclear magnetic resonance experiments were done as previously reported (9,28,58). Procedures involved rolling and inserting 0.5 cm wide leaf samples cut along the midrib into NMR tubes. Ice nucleation of the tissue was accomplished using ice crystals formed on a glass rod dipped in liquid nitrogen. Free induction decay was measured 20 microseconds after the second and each subsequent pulse. Pulses were 3 seconds apart to prevent saturation. Nuclear magnetic resonance signals were multiplied by temperature (°K)/273 to approximate the Boltzmann temperature correction.

Killing temperatures were based on solute leakage from frozen leaves (58,91,92). Frozen leaf samples were removed from the freeze chamber at various test temperatures and allowed to thaw at room temperature. Electrolyte leakage was determined as previously reported (91), except the incubation interval was expanded to 24 hrs and the leaves were sectioned into 1 cm strips. Typically, undamaged plant tissue yields about 5-10% of the electrolytes while severely damaged tissue yields about 80% electrolyte loss. A plot of percent conductivity vs. temperature yields a sigmoid curve with killing temperature at the inflection point.

Osmotic potential was determined psychrometrically. Expressed cell sap from each species was used to determine osmotic potential using a Wescor osmometer. The cell sap was obtained by placing leaves killed in liquid nitrogen in the barrel of a 5 ml syringe. The syringe

was placed in a 50 ml centrifuge tube and spun at 48,000 g for 20 minutes at 4°C. Water content was determined using fresh and oven-dried tissue weights [(fresh wt - dry wt)/dry wt].

Results and Discussion

Killing temperatures for lemon, grapefruit, orange, and mandarin were -4, -4, -7, and -11°C, respectively (Table 1). These values are consistent with previous cold hardiness ratings of citrus trees. For example, 'Star Ruby' grapefruit trees on sweet orange rootstock had 59% leaf kill at -4.4°C (105). 'Valencia' orange seedlings survived -6.7°C following controlled acclimation and were more cold hardy than 'Duncan' grapefruit and 'Rough' lemon (100). 'Clementine' mandarin was more cold hardy than 'Valencia' and 'Navel' orange, 'Ruby' and 'Marsh' grapefruit, and 'Mexican' lime trees following the 1962 freeze (111).

Citrus cold hardiness depends on temperature acclimation. The relative cold hardiness of species appears to be consistent from study to study. This is probably due to differences in threshold growth cessation temperatures since citrus plants do not attain rest (true dormancy). Mandarins stop growing at a higher temperature than orange and grapefruit which cease growth at a higher temperature than lemon and lime (118). The citrus shoots used in this study came from trees in the same plot; hence, they were exposed to similar hardening conditions.

The liquid water content (g H_2O/g dry wt) of unfrozen samples (L_0) was not significantly different ($\alpha = .05$) (Table 1). Also, the amount of unfreezable water (k) was not related to hardiness, just as

Table 1. Cold hardiness, water status, and melting point depression of citrus leaves

Citrus variety	Killing temperature (°C) K_T	Liquid water at 0°C[z] L_0	Liquid water at -10°C[z]	Fraction liquid water at K_T $\frac{L_{K_T} - k}{L_0 - k}$	Unfreezable water[z] k	ΔT_m (°C) psychrometry[x]	ΔT_m (°C) NMR[y]
Lemon	-4	1.26 ± .039 a	.64	.95	.114	1.44 ± .054 a	4.57 ± .050
Grapefruit	-4	1.29 ± .026 a	.54	.76	.074	1.83 ± .070 a	4.14 ± .069
Orange	-7	1.33 ± .052 a	.54	.53	.055	2.09 ± .038 a	4.12 ± .063
Mandarin	-11	1.25 ± .031 a	.56	.31	.195	1.64 ± .116 a	4.02 ± .091

[z] g H_2O/g dry wt

[y] values from regression of $L_T = (L_0 - k)\Delta T_m/T + k$ from -7 to -40°C

[x] means ± one standard deviation

in wheat (28,58). Equilibrium freezing studies show that a small amount of water remains unfrozen even at temperatures below -40°C (9, 28).

Melting-point depression, ΔT_m, of expressed sap was not significantly different among species. Melting-point depression, ΔT_m (°C), is directly related to osmotic potential, π(MPa), in dilute solutions

$$\Delta T_m = -224/T$$

where T is the absolute temperature. Melting-point depression and osmotic potential are a function of the concentration of solute. The temperature required to form a given amount of ice is lowered with increasing solute concentration. Therefore, plant freezing stress could be reduced by a reduction in ice formation (freeze avoidance) as a result of increased solute concentration. Differences in cold hardiness between citrus species could not be explained by differences in freeze avoidance as a result of dissolved solutes.

Freezing curves were similar for the four citrus species (Figures 1-4). Unfrozen water at -10°C ranged from .54 to .64 g/g dry wt with no apparent relationship to hardiness (Table 1). This represents 41-51% liquid water at -10°C. These are much higher values than those reported for Solanum species (15-22%) and cereals (23-37%) at -10°C (9,28). The only parameter found to be correlated with frost hardiness was the amount of unfrozen water at the killing temperature [$r = .95$ for K_T vs. $(L_{K_T} - k)/(L_0 - k)$]. Thus, the more cold-hardy leaves survived freezing of a larger fraction of tissue water.

Figure 1. Amount liquid water, L_T(g H_2O/g dry wt), vs. temperature (°C) (a) and inverse temperature (b) for 'Lisbon' lemon. Dashed line represents ideal freezing behavior.

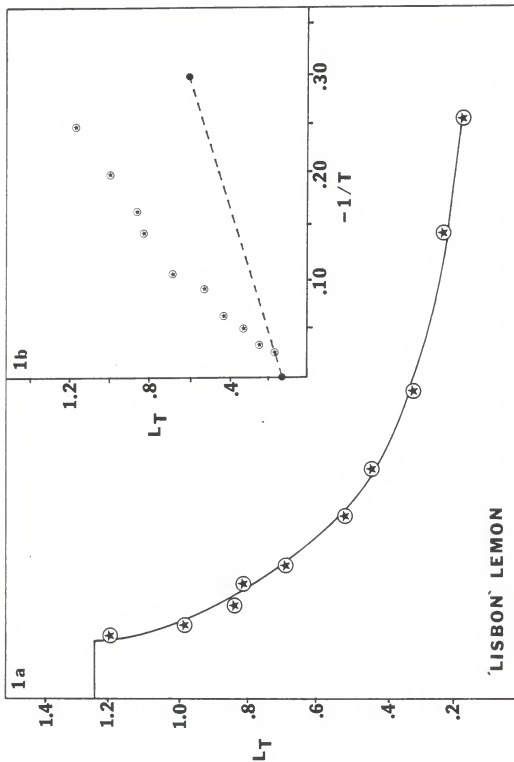

1a
1b
1.4
1.2
1.0
.8
.6
.4
.2
L_T
-10
-20
-30
-40
TEMPERATURE (°C)
'LISBON' LEMON
1.2
.8
.4
.10
.20
.30
-1/T

Figure 2. Amount liquid water, L_T(g H_2O/g dry wt), vs. temperature, (°C) (a) and inverse temperature (b) for 'Ruby Red' grapefruit. Dashed line represents ideal freezing behavior.

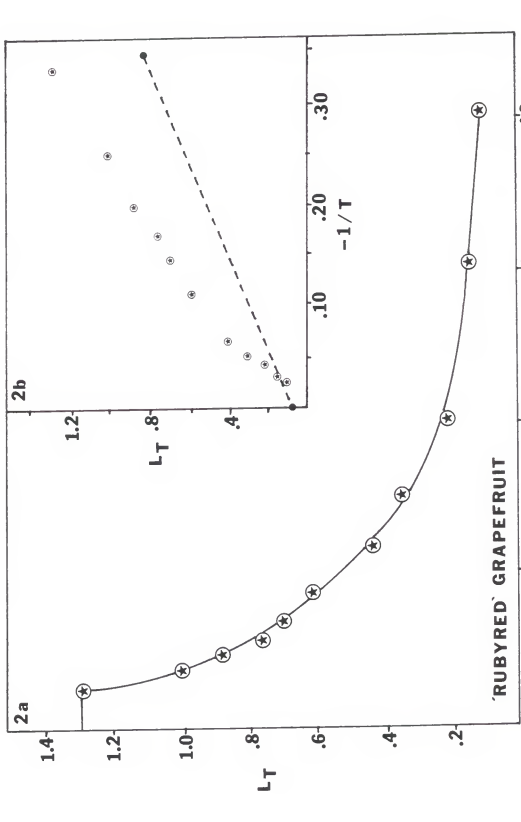

2a
2b
1.4
1.2
1.0
.8
.6
.4
.2
L_T
-10
-20
-30
-40
TEMPERATURE [°C]
'RUBYRED' GRAPEFRUIT
.10
.20
.30
-1/T

Figure 3. Amount liquid water, L_T(g H_2O/g dry wt), vs. temperature (°C) (a) and inverse temperature (b) for 'Valencia' orange. Dashed line represents ideal freezing behavior.

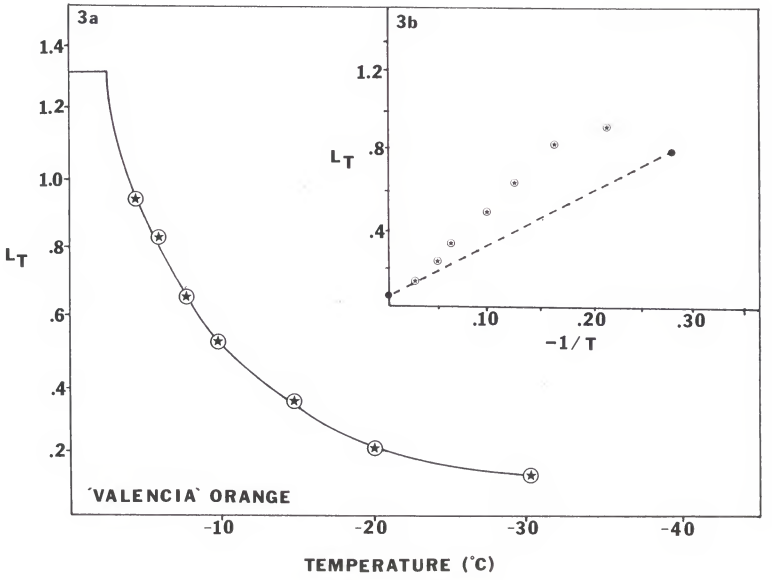
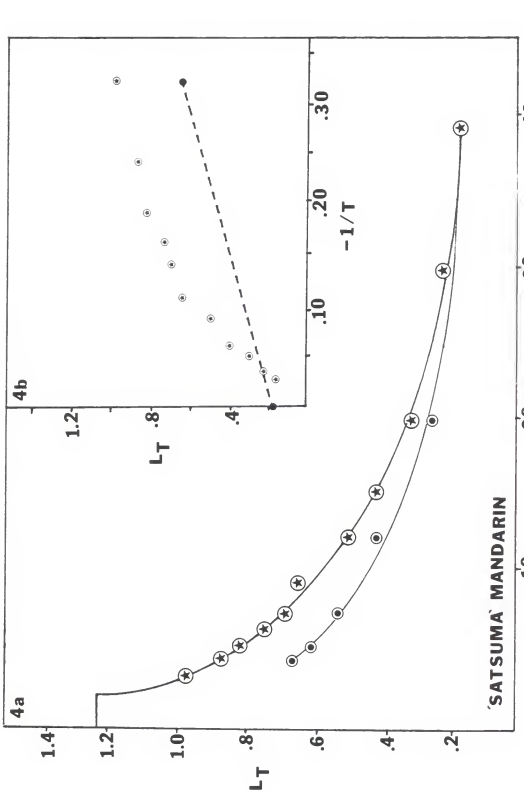

3a
3b
1.4
1.2
1.0
.8
.6
.4
.2
L_T
-10
-20
-30
-40
TEMPERATURE (°C)
'VALENCIA' ORANGE
1.2
.8
.4
L_T
.10
.20
.30
-1/T

Figure 4. Amount liquid water, L_T(g H_2O/g dry wt), vs. temperature (°C) (a) and inverse temperature (b) for 'Satsuma' mandarin. The lower curve (a) represents thawing and the dashed line (b) represents ideal freezing behavior.


4a
4b
'SATSUMA' MANDARIN
L_T
TEMPERATURE [°C]
-1/T
1.4
1.2
1.0
.8
.6
.4
.2
-10
-20
-30
-40
.10
.20
.30

Liquid water (L_T) vs. temperature [T(°C)] exhibits the following relationship (28,90):

$$L_T = (L_0 - k)\Delta T_m/T + k$$

The plot of L_T vs. -1/T is linear with slope $(L_0 - k)\Delta T_m$ and intercept k where L_0 is the liquid water at 0°C, ΔT_m is the average melting point depression and k is the unfreezable water (28). Values for ΔT_m determined in this manner for citrus are considerably larger than ΔT_m determined psychrometrically. Other studies comparing ΔT_m obtained by the two methods usually show values from NMR freezing curves to be about 1.5 times larger than psychrometric values (58,66).

Plots of the amount liquid water vs. temperature (°C) resulted in hyperbolas [Figures 1(a)-4(a)]. This relation was also found in cereal crowns (28) and Solanum species (9). In these studies, liquid water vs. inverse temperature yielded a straight line. In the case of shagbark hickory, which contains a deep supercooled fraction, the inverse temperature plot is not linear (5). There is little freezing until the homogeneous ice nucleation temperature is reached. This results in much larger L_T values than for samples that freeze ideally. Inverse temperature plots of citrus are intermediate between the ideal freezing and the deep supercooling cases [Figures 1(b)-4-(b)]. There was a reduction in ice formation that could not be accounted for by osmotic properties such as ΔT_m. The reduced ice formation was not evident during thawing of the tissue [Figure 4(b)]. This indicates a freeze hysteresis of the cells. Exposure to

temperatures far below the killing range apparently disrupted the cells, resulting in a curve that more closely resembles ideal freezing.

The observed reduction in ice formation during freezing may be the result of pressure potential. Pressure potential may play an important role in cell water relations at freezing temperatures. Negative pressure potential can result in a reduction in ice formation. The basis of negative pressure in a cell exposed to extracellular ice must be in the resistance to collapse of the entire cell which is mainly a result of cell wall rigidity. A pliable cell wall will collapse more easily resulting in near-ideal freezing behavior. A very rigid wall will resist collapse, causing a negative pressure potential and reduced ice formation.

To test the hypothesis that negative pressure potential affects freezing behavior in citrus leaves, tissue samples of lemon and mandarin were plunged into liquid nitrogen prior to insertion in the spectrometer chamber at -3°C. This treatment was designed to freeze the cells intracellularly and eliminate pressure potential contribution to water potential. This treatment had a significant effect on freezing curves, reducing the amount of liquid water. This caused ΔT_m values from regression to be lower for mandarin and substantially lower for lemon (Table 2). Therefore, pressure potential appears to account for a portion of the cell's water potential at freezing temperatures.

Studies comparing the fraction of unfrozen water in intact tissue and in expressed sap at freezing temperatures provided additional support for the negative pressure potential hypothesis. Pressure

Table 2. Freezing parameters for prefrozen (-196°C) citrus leaves

	Prefrozen leaves	
Citrus variety	Unfreezable water[z] k	ΔT_m (°C) NMR[y]
Lemon	.179	2.88
Mandarin	.177	3.73

[z] g H_2Og dry wt

[y] values from regression of $L_T = (L_0 - k)\Delta T_m/T + k$ from -4 to -12°C

potential arising from the cell wall is not present in the latter case. Lemon tissue was treated as in the previous section, but the freezing curve was expressed as the fraction of unfrozen water $(L_T - k)/(L_0 - k)$ vs. $-1/T$ rather than as the amount of unfrozen water (g H_2O/g dry wt) to facilitate comparison with expressed sap. Leaves were frozen in liquid nitrogen, thawed and centrifuged at 48,000 g for 20 minutes at 4°C through glass wool to obtain the tissue solution. The amount of extracellular water has been assumed to be negligible resulting in minimal dilution effects. The expressed sap was frozen and the freezing curve expressed as the fraction of unfrozen water at 0°C. The regression equation for intact tissue was found to be $f(x) = 3.80x$ ($r^2 = .98$) and $f(x) = 2.54x$ ($r^2 = .99$) for the tissue solution. Clearly, there is a reduction in ice formation in the intact tissue as evidenced by the greater slope (slope = ΔT_m).

If care is taken to ice nucleate samples at warm temperatures, the freezing temperature of tolerant species is clearly different from the killing temperature (Table 1, Figures 1-4). In fact, species differing in hardiness exhibit similar freezing behavior. Differences in hardiness between citrus species are best explained by differences in the amount of frozen water tolerated at the killing temperature.

Freezing curves for citrus species were unusual because less ice was formed than expected for ideal freezing behavior. Freezing curves can be explained by the formation of negative pressure potential during freezing.

CHAPTER II
THE ROLE OF ICE NUCLEATION ACTIVE BACTERIA IN FROST INJURY TO TENDER PLANTS

Introduction

Some crop plants cannot tolerate ice formation in the tissue; hence, the only protective mechanism for this type of plant is freeze avoidance (3,49,59,60). These sensitive plants may supercool in the absence of heterogeneous ice nuclei such as ice nucleation active (INA) bacteria to temperatures below those normally experienced during frost (52,60). Lindow et al. (50) and Marcellos and Single (58) observed supercooling to -10°C in corn and wheat. Present evidence indicates Pseudomonas syringae and Erwinia herbicola are INA bacteria and trigger frost injury in tender plants by serving as ice nucleating agents (3,49, 52,56,95). Pseudomonas syringae is ubiquitous, infecting a broad spectrum of host plants (14). It is found in residence on plants void of pathological symptoms (46,80,81) and in numbers sufficiently high to account for ice nucleation during winter months (49).

Bacteria active in ice nucleation were detected in 74 of 95 plant species surveyed (49). Changes in the nucleating ability of leaf material have been attributed to P. syringae (95) which parallel the total bacterial population (49). Schnell and Vali reported ice nucleation by P. syringae at -1.3°C using a bacterial suspension-droplet technique in which droplets were cooled on a refrigerated thermo-electric plate (82).

Repeated freezing of the same drops often resulted in a different freezing sequence with single drops differing in freezing temperatures by as much as 5°C (56).

Objectives were to determine whether tender plants avoid frost injury by supercooling and if INA bacteria limit supercooling.

Materials and Methods

Tomato (Lycopersicon esculentum Mill. 'Walter'), soybean (Glycine max. L. 'Bragg'), pepper (Capsicum annuum L. 'Calwonder'), begonia (Begonia semperflorum L. 'Vodka'), coleus (Coleus blumei L. 'Sabermix'), marigold (Tagetes spp. L. 'Giant Fluffy'), zinna (Zinnia spp. L. 'Old Mexico'), and calendula (Calendula officinalis L. 'Pacific Beauty') plants were grown in a mixture of 3 peat: 2 sand: 1 bark chips in metal flats. Part of the plants used (for leaflet inoculation experiments) were grown in a greenhouse and the remainder in modified growth chambers under a 12-hr photoperiod. Temperature and humidity in growth chambers were maintained at 24 ± 2°C and 60 ± 10% relative humidity and fluctuated with ambient winter conditions in the greenhouse. Test plants were either sprayed at a rate of about 0.5 ml per plant with a water suspension of P. syringae or water (control) or inoculated with a cotton swab on the underside of a leaflet.

Bacteria were cultured in a medium containing 1% dextrose, 1% Bacto-peptone, and 0.1% Bacto-casamino acids at 30°C unless specified otherwise. The pellet was suspended in sterile deionized water after centrifugation of cultures in the late log phase of growth. The suspension was adjusted to 0.3 (optical density) at 600 nm which

corresponds to about 4 x 10^8 cells/ml. Shoots about 10 cm in length were placed in 25 x 200 mm tubes and submerged in a 190 ℓ refrigerated glycol bath. Temperature fluctuation was ±0.2°C as monitored by a thermocouple in a test tube. Plants were held at each test temperature for 1 hr and then examined for nucleation. Freezing was determined visually by water-soaking and loss of turgor upon thawing.

Results and Discussion

Cold-hardy leaves become water-soaked but are not killed upon freezing. Therefore viability tests such as electrolyte leakage or regrowth must be utilized to determine frost damage. Tender plants, on the other hand, are killed when frozen. Tender plants become water soaked and lose turgor upon thawing. These visual tests were found to be in excellent agreement with electrolyte leakage viability tests (Table 3). Tomato shoots were sprinkled with ice (or untreated) to verify that freezing, not low temperature, causes damage. At temperatures between -2.5 and -6.0°C, only those plants nucleated with ice were frozen. All plants were frozen at -8.0°C and below (Table 4). It is likely that intrinsic nucleators are effective at the latter temperature. The lack of freezing above -2.0°C may be due, in part, to the freezing point depression of the tissue solution. From these data it was concluded that tender plants such as tomato and soybean are killed when frozen. Frost-killed plants are readily apparent by visual observation.

Tomato plants that were sprayed with P. syringae froze and were killed above -4°C while control plants without INA bacteria had 58% of

Table 3. Leakage of electrolytes and appearance of water-soaking (+) in soybean shoots following exposure to low temperature stress

Temperature (°C)	'Bragg' Soybean	
	Electrolyte leakage (%)	Water-soaking
-3	4	-
-4	3	-
-5	6	-
-6	4	-
-7	5	-
-8	3	-
-9	60	+
-10	63	+
-11	61	+

Table 4. Leakage of electrolytes from tomato shoots following low temperature stress. Ice was used to nucleate one-half of the samples at each temperature (*)

	Tomato shoots	
Temperature (°C)	Nucleation (*)	Electrolyte leakage (%)
Control		11
-0.5		17
-0.5	*	13
-1.0		19
-1.0	*	11
-1.5		17
-1.5	*	15
-2.0		11
-2.0	*	13
-2.5		15
-2.5	*	55
-3.0		14
-3.0	*	69
-4.0		13
-4.0	*	88
-6.0		14
-6.0	*	72
-8.0		67
-8.0	*	67
-10.0		64
-10.0	*	69

the plants supercooling and surviving -7°C (Table 5). Similar results are presented for soybean, pepper, begonia, marigold, calendula, and coleus plants in Tables 6-11. Erwinia herbicola was also found to be an efficient ice nucleator (Table 12). Pseudomonas syringae inoculated and control tomato shoots were crushed in 5 ml sterile deionized water and droplets of these suspensions were frozen on a Peltier cooling plate. Freezing temperatures of 20 µl drops were -4.2 ± 1.4°C and -13.4 ± -2.2°C for inoculated and control suspensions, respectively. Thus, the INA bacteria inoculated suspensions froze over a slightly lower temperature interval compared to the INA bacteria inoculated tomato shoots. This effect was more pronounced with the control tomato suspensions and shoots. This behavior was expected due to sequestering of nuclei in smaller volume drops compared to whole shoots (94).

Tomato plants were sprayed with 4×10^8 cells/ml of P. syringae and frozen over time to determine the persistence of bacterial ice nucleation. The most effective interval was 24 hrs after inoculation (Figure 5). The temperature required to freeze one-half of the plants decreased with time, although inoculated plants froze at a higher temperature than control plants even after 408 hrs.

Tomato leaves were inoculated with 6 concentrations of P. syringae with a cotton swab 24 hrs. prior to freezing to determine the threshold inoculum concentration necessary for ice nucleation. Efficient ice nucleation occurred at an inoculum concentration of 4×10^5 cells/ml; 80% of the leaves inoculated with this concentration were frozen at -4°C, as compared to only 7% of the control leaves

Table 5. Effect of inoculation with 4 x 10^8 cells/ml *P. syringae* on frost injury to tomato shoots

Temperature (°C)	Tomato plants frozen (%)	
	Water-sprayed control	Sprayed with *P. syringae*
-3	0	0
-4	1	93
-5	5	100
-6	17	100
-7	42	100
-8	69	100

Table 6. Effect of inoculation with 4 x 10^8 cells/ml *P. syringae* on frost injury to soybean shoots

Temperature (°C)	Soybean plants frozen (%)	
	Water-sprayed control	Sprayed with *P. syringae*
-3	0	0
-4	0	19
-5	3	51
-6	6	66
-7	15	75
-8	48	87
-9	84	96
-10	95	100

Table 7. Effect of inoculation with 4 x 10^8 cells/ml *P. syringae* on frost injury to pepper shoots

Temperature (°C)	Pepper plants frozen (%)	
	Water-sprayed control	Sprayed with *P. syringae*
-3	0	4
-4	3	84
-5	11	100
-6	50	100
-7	81	100
-8	87	100

Table 8. Effect of inoculation with 4 x 10^8 cells/ml *P. syringae* on frost injury to begonia shoots

Temperature (°C)	Begonia plants frozen (%)	
	Water-sprayed control	Sprayed with *P. syringae*
-3	0	0
-4	0	11
-5	0	34
-6	14	57
-7	57	79
-8	91	98

Table 9. Effect of inoculation with 4 x 10^8 cells/ml *P. syringae* on frost injury to marigold shoots

Temperature (°C)	Marigold plants frozen (%)	
	Water-sprayed control	Sprayed with *P. syringae*
-3	1	1
-4	2	96
-5	8	99
-6	47	100
-7	93	100
-8	100	100

Table 10. Effect of inoculation with 4 x 10^8 cells/ml *P. syringae* on frost injury to calendula shoots

Temperature (°C)	Calendula plants frozen (%)	
	Water-sprayed control	Sprayed with *P. syringae*
-3	5	15
-4	5	90
-5	5	100
-6	10	100
-7	35	100
-8	55	100

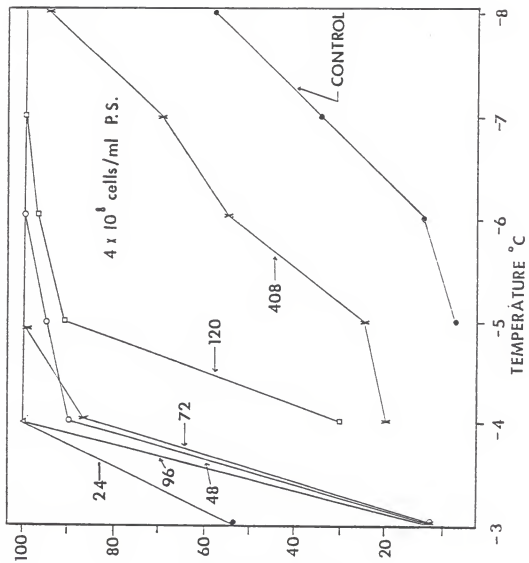
Table 11. Effect of inoculation with 4 x 10^8 cells/ml *P. syringae* on frost injury to coleus shoots

Temperature (°C)	Coleus plants frozen (%)	
	Water-sprayed control	Sprayed with *P. syringae*
-3	0	0
-4	3	23
-5	10	49
-6	18	59
-7	50	75
-8	74	82

Table 12. Effect of inoculation with 4 x 10^8 cells/ml *E. herbicola* on frost injury to tomato shoots

Temperature (°C)	Tomato plants frozen (%)	
	Water-sprayed control	Sprayed with *E. herbicola*
-3	1	1
-4	6	65
-5	12	96
-6	29	100
-7	49	100
-8	77	100

Figure 5. Percentage of tomato plants frozen from 24 to 408 hrs after inoculation with 4 x 10^8 cells/ml *P. syringae*. Results from control plants have been summarized as means at each temperature.


% PLANTS FROZEN
100
80
60
40
20
-3
-4
-5
-6
-7
-8
TEMPERATURE °C
4 x 10⁸ cells/ml P.S.
CONTROL
24
48
72
96
120
408

(Table 13). In a similar experiment, zinnia plants were sprayed with tenfold dilutions of a bacterial suspension ranging from 4×10^4 to 4×10^8 cells/ml. Once again, a threshold inoculum concentration of about 4×10^5 cells/ml was necessary for high-temperature ice nucleation (Table 14). Freezing percentages increased with increasing inoculum concentration from 4×10^5 to 4×10^8.

Different-size areas of tomato leaflets (one segment of the compound leaf) were inoculated with a suspension of 4×10^8 cells/ml P. syringae using a cotton-tipped swab. All treatments were applied to each leaf, one per leaflet. A spot about 4 mm in diameter was sufficient to result in complete freezing of about 60% of the leaflets at -4°C. Inoculation of 0.25 or 0.5 of the leaf resulted in 70 and 90% of the leaflets frozen at -4°C, respectively. All of the leaves with 4 mm diameter spots of bacteria were frozen at -5°C (Table 15). It was concluded from these data that bacteria in sufficient numbers in a small area of a leaf will result in freezing of the entire leaf.

It appears that ice nucleation active bacteria could be a significant factor in frost susceptibility of tender plants.

Table 13. Effect of inoculating tomato leaflets with different concentrations of P. syringae (24 hrs prior to freezing) on frost injury

P. syringae concentration (cells/ml)	Tomato leaflets frozen (%)					
	Temperature					
	-3°C	-4°C	-5°C	-6°C	-7°C	-8°C
Control	0	7	7	13	33	54
4×10^3	0	7	20	27	60	93
4×10^4	0	0	10	20	40	60
4×10^5	0	80	93	93	100	100
4×10^6	0	93	93	100	100	100
4×10^7	7	87	100	100	100	100
4×10^8	7	87	100	100	100	100

Table 14. Frost injury to zinnia plants sprayed with different concentrations of P. syringae 24 hrs prior to freezing

P. syringae concentration (cells/ml)	Zinnia plants frozen (%)					
	Temperature					
	-3°C	-4°C	-5°C	-6°C	-7°C	-8°C
Control	0	0	0	5	45	75
4×10^4	0	0	5	10	20	40
4×10^5	0	30	55	60	75	85
4×10^6	10	35	75	90	95	95
4×10^7	0	55	90	100	100	100
4×10^8	0	95	100	100	100	100

Table 15. The effect of inoculating different-sized areas of the abaxial leaflet of tomato with 4 x 10^8 cells/ml P. syringae 24 hrs prior to freezing

Temperature (°C)	Leaflets frozen (%)				
	Area of tomato leaflet treated				
	None	4 mm diameter spot	0.25	0.5	Entire
-3	0	0	0	0	0
-4	0	60	70	90	100
-5	0	100	90	100	100
-6	0	100	100	100	100
-7	27	100	100	100	100
-8	60	100	100	100	100

CHAPTER III
FACTORS AFFECTING ICE NUCLEATION BY BACTERIA

Introduction

Pure water melts at 0°C but freezes at a lower temperature. The amount of supercooling is determined by the concentration and efficiency of ice nucleators present, the sample size, and (to a lesser degree) the cooling rate (94,96). The lower limit of supercooling for pure water is about -40°C (5,99). Freezing point depression from dissolved solutes is additive to the depression from supercooling (6, 73). A solution that is unfrozen below 0°C due to solutes is in a stable state, but a supercooled solution is metastable.

The phase transition from liquid water to solid ice may be viewed as a process involving two temperature-dependent stages. An ice nucleus must form in the liquid phase and then grow. An activation energy must be provided for a nucleus to form since entropy is decreased and an interface is formed (16,55). Assuming a spherical nucleus, the formation would involve a bulk free energy change which is negative below the melting point as well as an interfacial change in free energy which is always positive (4). Bulk free energy is a function of the radius cubed while the interfacial free energy is a function of the square of the radius. Therefore, as the radius of the nucleus increases, the bulk free energy term becomes negative at a faster rate than the interfacial free energy term becomes more positive. A critical radius

is reached at which continued growth results in a decrease in free energy and growth becomes spontaneous. The critical radius is smaller at lower temperatures. Growth rate of a nucleus will be determined by the driving force (which is the difference in free energy between supercooled water and ice at the same temperature) and the rate of diffusion.

Similar energy considerations hold for heterogeneous nucleation. However, instead of the chance aggregation of water molecules forming the nucleus as in homogeneous nucleation, suspended impurities or surfaces catalyze the formation of an ice nucleus. Heterogeneous nucleators act as templates for nucleus formation with the efficiency depending on the number of water molecules ordered into the crystal structure of ice.

Heterogeneous nucleators have been the subject of interest to cloud physicists. Their studies have been motivated, in part, by the prospect of modifying precipitation processes for man's benefit. Many substances (most notably silver iodide and clays of the kaolinite type) catalyze ice formation above -10°C. An unidentified ice nucleus active at -4°C was collected from a cloud by Kassander and coworkers in 1955 (42). Vali and coworkers later found that decaying leaf litter was a source of nuclei active at this temperature (95). Epiphytic bacteria proved to be the heterogeneous nucleators responsible for high temperature ice nucleation. This finding has become even more important since these bacteria have been implicated as causal agents of frost damage to tender plants.

Little research has been done on the factors affecting ice nucleation by bacteria. Maki et al. reported that ice nucleation by P. syringae was a dynamic property, fluctuating by as much as 5°C (56). They found that high temperature ice nucleation was reversibly lost when cultures grown at 20-5°C were no longer aerated. Cultures of P. syringae grown at 22-24°C were more active than cultures grown at lower or higher temperatures (51). The nucleation efficiency of Erwinia herbicola was reported to be affected by the culture medium with high sugar concentration favoring high temperature ice nucleation.

The objective of this research was to characterize ice nucleation properties of P. syringae, an INA (ice nucleation active) bacterium.

Materials and Methods

A Pseudomonas syringae van Hall (isolate C-9) culture was obtained from R. Schnell. Bacteria were cultured at 30°C (unless specified otherwise) in a medium containing 1% Bacto-peptone, 1% dextrose, and 0.1% Bacto-casamino acids. Flasks were held on an orbital shaker at 100-25 rpm to insure aeration. Cultures late in the log phase of growth (about 24 hrs) were centrifuged at 2000 g for 10 minutes. The bacterial pellet was suspended in sterile water and adjusted to 0.3 absorbance (about 4×10^8 cells/ml).

Freezing experiments were conducted on a greased thermoelectric cooling plate. A refrigerated glycol bath was circulated within the plate as a heat-sink. Plate temperature was monitored by .075 mm copper-constantan thermocouples. An automatic pipette was

used to deliver uniform 20 µl drops. Treatments were partially randomized on the plate (all drops within a treatment were grouped to facilitate observation but treatments were randomly assigned to sectors of the plate). Frozen drops were detected visually by their milky appearance.

A 4 x 10^8 cells/ml water suspension of P. syringae grown at 22°C was used in oxygen depletion studies. The system (Yellow Springs Instrument Company, Model 53) was held at 22°C by a circulating water bath. Percentage oxygen saturation in the sample vial and median freezing temperature of drops of the bacterial suspension were measured over the course of five hours. Samples were drawn with a long-needled syringe through the same channel on the side of the probe used to eliminate gas bubbles.

Results and Discussion

Ice nucleation efficiency of P. syringae is affected by the growth temperature with the 22-24°C range being optimum for the most active cultures (51). This finding was substantiated by freezing drops of cultures grown at 22 and 30°C. Freezing data include (1) the temperature at which the first drop froze (T_1); (2) the temperature at which half of the drops were frozen (T_{50}); and (3) the temperature at which all of the 20 µl drops had frozen (T_{100}) (Table 16). All of the drops from the 22°C culture were frozen by -3.0°C, while none of the drops from the 30°C culture froze until -7.7°C. Low-efficiency cultures grown at 30°C were refrigerated at 5°C to determine if static cultures could be activated (little or no growth is expected at 5°C).

Table 16. Freezing temperatures of 20 µl drops [first (T_1), median (T_{50}), and last (T_{100})] of suspension of P. syringae (4×10^8 cells/ml) grown at 22° and 30°C and water

	22°C		30°C	
Temperature	H_2O	P. syringae	H_2O	P. syringae
T_1	-8.1	-1.6	-7.4	-7.7
T_{50}	-11.9	-2.2	-11.8	-8.3
T_{100}	-14.7	-3.0	-14.6	-10.5

Two hours at 5°C dramatically increased the nucleation efficiency of the P. syringae suspension (Figure 6). Nucleation temperatures were increased by as much as 5°C. A concentration of about 4×10^5 cells/ml was the threshold necessary for high temperature nucleation of the activated (chilled) culture. This concentration yields 8×10^3 bacteria per drop. It appears that not every cell is active in ice nucleation.

Maki et al. (56) attributed the high temperature ice nucleation of P. syringae to aeration of the cultures (grown at 20-25°C). They reported this phenomenon to be reversible. This finding seems consistent with chilling effects since oxygen is more soluble at the lower temperature. However, similar results were not obtained by this researcher. A P. syringae culture grown at 22°C was diluted to 4×10^6 cells/ml. The sample was divided into two flasks, one of which was aerated by tubing from a standard laboratory compressed-air fixture. Aeration did not stop the culture from losing efficiency at an ambient temperature of 26°C (Table 17).

Oxygen electrode studies are not consistent with the hypothesis that the availability of oxygen determines the efficiency of bacterial ice nucleation. A suspension of P. syringae grown at 22°C (4×10^8 cells/ml) was placed in an oxygen electrode chamber. A standardized probe was positioned in the suspension such that air bubbles were excluded. The percentage oxygen saturation was measured over time as bacteria depleted the available oxygen. Samples for freezing point determinations were drawn with a long-needled syringe through the channel

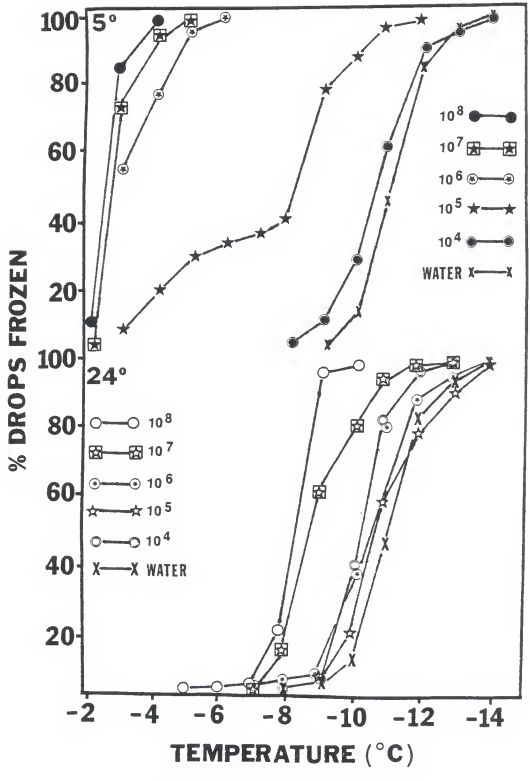


Figure 6. Freezing percentages of drops of P. syringae grown at 30°C. Suspensions (10^4 to 10^8 cells/ml) were held at 24° or 5°C for 2 hrs following resuspension in water.

Table 17. Freezing temperatures of aerated and non-aerated drops of 4×10^6 cells/ml P. syringae. Freezing temperatures of the first (T_1), median (T_{50}), and last (T_{100}) drops were determined at 0 and 3 hrs after suspension in water

	Freezing temperatures of drops (20 µl)			
	0 hrs		3 hrs	
Temperature	H_2O	P. syringae	Non-aerated	Aerated
T_1	-7.5	-2.1	-6.5	-6.8
T_{50}	-11.2	-2.7	-7.4	-7.4
T_{100}	-14.0	-4.2	-8.1	-8.0

on the side of the probe (designed to eliminate air bubbles). Even though the oxygen saturation was reduced to about 10% in an hour and remained low, the median freezing temperature of drops sampled from the chamber did not decrease appreciably (Table 18).

In another experiment, a P. syringae culture was grown at 30°C to produce low-efficiency ice nucleation. Drops from this suspension were frozen and then the sample was divided into 3 aliquots. One was held at room temperature (24°C), another was placed in a refrigerator at 5°C, while the third had a layer of mineral oil added over the top to exclude air before chilling. Both chilled cultures increased markedly in freezing efficiency (Table 19). These experiments indicate that oxygen concentration does not directly influence the ice nucleation efficiency of P. syringae.

A P. syringae culture was grown at 22°C and allowed to sit at ambient temperature (24°C). The freezing temperature of 20 µl drops was measured over an interval of about 24 hrs. The culture was then split into two samples. One was held at ambient temperature while the other sample was switched back and forth between chambers at 5 and 30°C. The freezing temperatures of the ambient sample slowly decreased for the duration of the experiment (Table 20). After 24 hrs, the sample was dilution plated and it was determined that the bacterial concentration had not changed from 4×10^8 cells/ml. Each time the sample was held at 30°C, the freezing temperature dropped and when the sample was held at 5°C the freezing temperature increased. Thus, the temperature effects on bacterial ice nucleation are reversible.

Table 18. Percentage oxygen saturation and median freezing temperature (°C) of drops from a P. syringae suspension

	P. syringae suspension	
Time (hrs)	% Saturation	T_{50}
0	94	-2.1
1	11	---
2	10	-2.3
3	9	-2.3
5	9	-2.4

Table 19. Freezing temperatures of 20 µl drops of P. syringae suspensions (4×10^8 cells/ml) held at ambient temperature (24°C), chilled (5°C), or chilled after a layer of mineral oil was placed over the suspension

Median freezing temperatures (°C)							
0 hrs		2 hrs			4 hrs		
H_2O	P. syringae	Ambient	Chilled	Chilled with oil	Ambient	Chilled	Chilled with oil
-12.7	-7.9	-7.3	-2.9	-3.7	-6.7	-2.8	-3.2

Table 20. Freezing temperatures of P. syringae drops from cultures held at ambient temperature (24°C) or switched back and forth from incubation at 5° and 30°C

Temperatures	0 hrs	9 hrs	22 hrs	29 hrs		35 hrs		47 hrs		54 hrs	
	Ambient	Ambient	Ambient	Ambient	30°C (4 hrs)	Ambient	5°C (6 hrs)	Ambient	30°C (12 hrs)	Ambient	5°C (7 hrs)
T_1	-1.7	-2.7	-4.3	-5.4	-6.9	-6.3	-3.1	-6.9	-6.4	-7.2	-2.7
T_{50}	-2.5	-2.9	-7.5	-7.8	-8.4	-8.0	-4.1	-7.9	-8.4	-8.2	-4.2
T_{100}	-3.0	-3.8	-8.3	-8.4	-8.5	-8.7	-6.6	-8.7	-9.1	-9.1	-6.2

To determine the optimum storage temperature for ice nucleation, bacteria were grown at 22°C and held at temperatures ranging from 34 to -15°C. Temperatures between 5 and 14°C resulted in highest freezing temperatures (Table 21). Once again, the loss of efficiency was reversed by holding the samples at 5°C.

Experiments where concentration series of bacteria have been frozen indicate that bacterial ice nucleation is a threshold phenomenon (56, Figure 6). About 10^5 cells/ml are necessary which corresponds to about 10^4 cells for the drop volumes used. This indicates that all cells do not express the ice nucleation character, at least not continuously. If 1 in 10^4 cells is active, then by selection a culture with all cells active could be obtained. A culture without ice nucleation activity would be relatively easy to obtain. This proved to be an unsatisfactory approach. When the drop freezing at the lowest temperature was used to initiate a new culture, loss of ice nucleation was not observed (Table 22). The last of 60 drops from a 4×10^5 cells/ml suspension froze at -16.9°C (while the first drop froze at -3.0°C). The drop which froze at -16.9°C was thawed and recooled. It remained unfrozen at -16.9°C indicating a short-term stability. This drop was used to initiate a new culture of P. syringae. The high and low freezing temperatures of a 4×10^5 suspension of this "first generation" culture were -7.2 and <-16.7°C. The low drop (-16.7°C) was thawed and recooled, remaining unfrozen at -17.6°C. This drop was used to initiate a "second generation" culture which yielded high and low freezing temperatures of -2.4 and <-16.9°C. This procedure was

Table 21. Freezing temperatures of 20 µl drops of P. syringae held at various temperatures. All samples were placed at 5°C after 3 hr measurement

	0 hrs	
Temperature	P. syringae 4×10^6	Tap H_2O
T_1	-2.3	-10.4
T_{50}	-2.7	-13.2
T_{100}	-3.6	-15.7

	1.5 hrs						
Temperature	34°C	30°C	Ambient	22°C	14°C	5°C	-15°C
T_1	-7.9	-3.3	-2.4	-2.1	-2.1	-2.3	-2.4
T_{50}	-10.2	-8.0	-7.7	-2.8	-2.7	-2.8	-2.9
T_{100}	-16.2	-8.5	-8.4	-4.7	-3.2	-3.3	-4.8

	3 hrs						
Temperature	34°C	30°C	Ambient	22°C	14°C	5°C	-15°C
T_1	-8.6	-6.1	-7.2	-1.9	-2.3	-2.2	-2.5
T_{50}	-15.7	-7.9	-7.8	-2.9	-2.7	-2.5	-3.1
T_{100}	-16.5	-8.6	-8.6	-7.9	-3.1	-3.0	-5.1

—All samples placed at 5°C—

	24 hrs						
Temperature	34°C	30°C	Ambient	22°C	14°C	5°C	-15°C
T_1	-2.4	-2.2	-2.2	-2.0	-1.9	-2.1	-2.8
T_{50}	-3.0	-2.4	-2.6	-2.3	-2.5	-2.4	-3.6
T_{100}	-3.9	-2.8	-3.2	-3.0	-3.3	-3.0	-7.1

Table 22. Temperatures at which the first (T_1) of 60 drops froze and the last (T_{60}). P. syringae cultures were initiated from T_{60} of the previous generation and diluted to 4×10^5 cells/ml

Temperature	Initial culture		1st generation	2nd generation	3rd generation
	H_2O	P. syringae	P. syringae	P. syringae	P. syringae
T_1	-3.0	-3.0	-7.2	-2.4	-2.6
T_{60}	<-16.9	-16.9	<-16.7	<-16.7	<-16.7

followed to produce a "third generation" with very similar results. A drop that did not freeze at -16.7°C initiated a culture that froze as warm as -2.4°C. This reinforced the conclusion from chilling experiments that ice nucleation is a dynamic property of P. syringae.

Several drops were repeatedly frozen, thawed, and refrozen to determine the short-term temperature range of bacterial ice nucleation. The freezing sequence was virtually unchanged, once again indicating a short-term stability (Table 23). This experiment was repeated with the freezing order (highest to lowest) of 10 drops recorded through 10 freeze-thaw cycles. If cells of equal nucleating ability were uniformly distributed the mean freezing rank (sum of freezing orders/ number of cycles) for all drops would be clustered near the median. This was not observed (Table 24). One drop had a mean freezing rank of 1.1 which means that it was the first drop to freeze in every cycle except 1 (in which it was the second drop to freeze). The relative freezing temperature of a sample of drops seems to be stable in the short term and it appears that there is some variability in nucleation efficiency of a population of bacteria.

Over longer time intervals the nucleating ability is unstable, losing efficiency when the storage temperature is out of the 5-14°C range. Since the efficiency decline is not the result of mortality there are two explanations. Either a cell could be active at a progressively lower temperature or it could "shut off" entirely. In the latter case the sample would freeze at the temperature at which the next-most-active cell causes ice nucleation. To determine the

Table 23. Freezing temperatures of 20 µl drops of P. syringae (4×10^6 cells/ml) repeatedly frozen and thawed

Cycle #	Drop #					Sequence (highest to lowest)
	1	2	3	4	5	
1	-7.9	-8.1	-7.8	-7.6	-9.0	4,3,1,2,5
2	-7.4	-8.2	-7.3	-7.1	-8.8	4,3,1,2,5
3	-7.8	-7.9	-7.2	-7.3	-9.0	3,4,1,2,5
4	-7.7	-8.1	-7.2	-7.4	-8.8	3,4,1,2,5
Mean	-7.7	-8.1	-7.4	-7.4	-8.9	

Table 24. Mean rank (sum of freezing orders/number of cycles) of *P. syringae* drops (4×10^6 cells/ml) repeatedly frozen and thawed

Drop #:	1	2	3	4	5	6	7	8	9	10
Mean rank	4.3	6.8	6.9	2.8	4.3	6.9	5.8	7.4	5.1	1.1

Note: The freezing order (from highest to lowest) was recorded for each of 10 freeze-thaw cycles.

cause of this behavior the cumulative ice nucleation spectrum (CINS) derived by Vali (94) was employed. This equation determines the concentration of nucleators active at and above a particular temperature, independent of sample volume. Since the concentration of bacteria is known, the freezing behavior can be expressed as the number of cells/ice nucleus (cumulative ice nucleation frequency). If nucleation sites decay to progressively lower temperatures the CINF would shift to the right. The same concentration of nucleators would be present but active at a lower temperature. On the other hand, if sites turn off, the curve will shift up reflecting the "disappearance" of nucleators. A CINF for a culture of P. syringae grown at 30°C is shown in Figure 7. Data from many concentrations are pooled to obtain the curve. A culture of P. syringae was grown at 22°C and allowed to lose efficiency over time (held at 24°C). The freezing temperatures of 20 µl drops yielded the curves in Figure 8. The CINF curve shifts up as the culture loses efficiency indicating that cells "shut off." Chilling the culture resulted in a decrease in the number of cells/nucleus (an increase in the concentration of ice nucleators). The reversible "switching" behavior of bacteria active in ice nucleation may prove to be beneficial in the frost protection of tender plants if the mechanism is elucidated.

Figure 7. Cumulative nucleation frequency for 20 µl drops of a P. syringae culture grown at 30°C. Concentrations from 10^4 to 10^8 cells/ml were used.

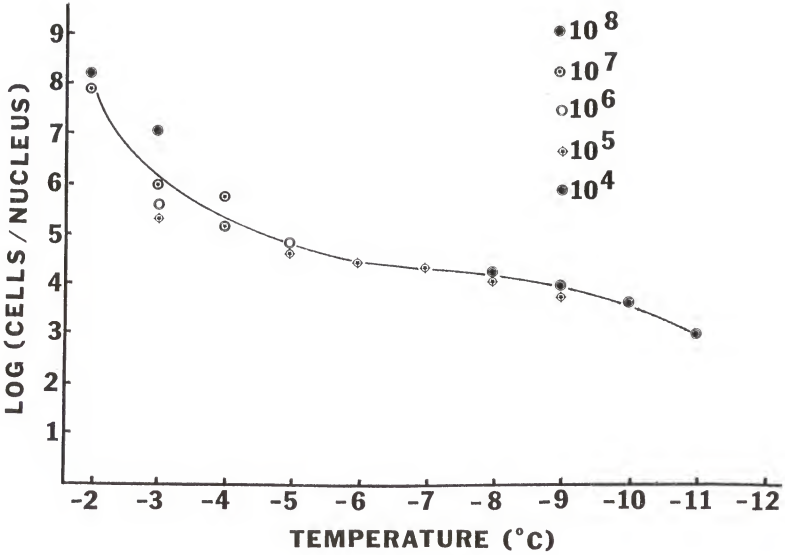
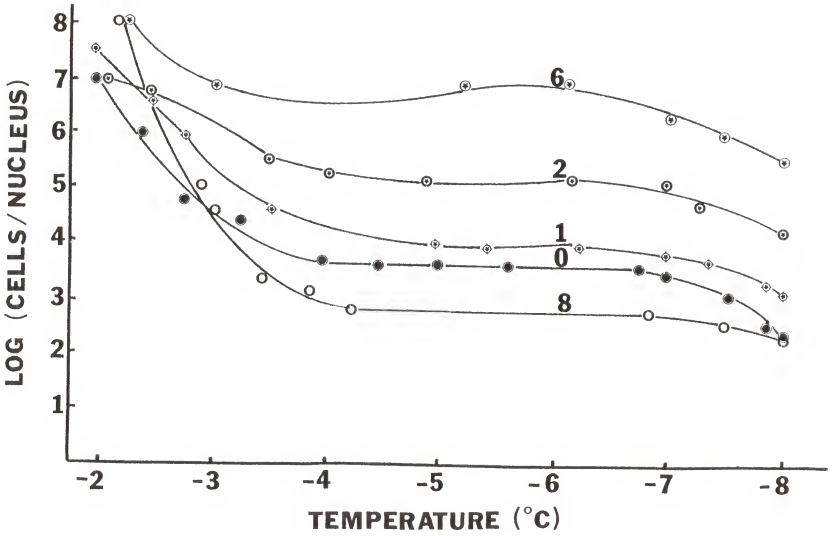

LOG (CELLS / NUCLEUS)
9
8
7
6
5
4
3
2
1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
TEMPERATURE (°C)
10 8
10 7
10 6
10 5
10 4

Figure 8. Cumulative nucleation frequency for a P. syringae culture held at 24°C for 6 hrs (curves 0-6) then placed at 5°C for 2 hrs (curve 8).


LOG (CELLS/NUCLEUS)
8
7
6
5
4
3
2
1
6
2
1
0
8
-2
-3
-4
-5
-6
-7
-8
TEMPERATURE (°C)

CHAPTER IV
REDUCTION OF BACTERIALLY INDUCED FROST DAMAGE

Introduction

Bacteria have been demonstrated to initiate frost damage to tender plants at relatively warm temperatures (3,49,52). Plants void of bacteria active in ice nucleation avoid frost damage by supercooling to as low as -10°C (52,49). Therefore, elimination of bacterial populations or negation of ice nucleation properties seems to be a logical approach to frost protection of tender plants.

Strategies for protection from bacterial ice nucleation include the use of antibiotics, bacteriophages, antinucleating compounds, and competitive bacteria. These methods have drawbacks. Antibiotic-resistant bacteria were described nearly 100 years ago by Kossiakoff [cited in Lowbury (54)]. Resistance to antibiotics is now routinely observed (2,15,20,87), partially due to the large number of organisms, their short generation time and the ability to exchange genetic material. Multiple resistance (to several antibiotics) has been observed in Escherichia coli (65), Serratia marcescens (43), and Pseudomonas aeruginosa (44). Resistant strains are now responsible for causing a significant portion of diseases that were previously caused by sensitive bacteria (86).

The use of phages has not proved to be highly successful in limiting bacterial populations. Disease control is possible only when

the phage is added to the bacterial suspension prior to inoculation (64). This may be the result of lack of contact between phage and bacterial cells. Okabe and Goto, in their extensive review article (64), state that absorption of the phage by all of the bacterial cells is not possible. They concluded that phages are of no value in controlling plant disease.

The objective of this research was to evaluate the use of certain antibiotics and competitive bacteria as agents to reduce bacterially induced frost damage.

Materials and Methods

Tomato plants (Lycopersicon esculentum Mill. 'Walter') were grown in a commercial potting mix in metal flats. The plants were kept in modified growth chambers under a 12 hr photoperiod. Temperature and humidity were maintained at 24 ± 2°C and 60 ± 10% relative humidity. Plants were sprayed with a water suspension of bacteria or water (control) at a rate of about 0.5 ml per plant. Bacterial cultures were obtained from R. Schnell [Pseudomonas syringae van Hall (isolate C-9)], Microlife Technics [Erwinia herbicola (Lohnis) Dye (isolates 26 and M232A)], and Abbott Laboratories [Bacillus spp. (isolate 13)]. Bacteria were cultured at 30°C in a medium containing 1% Bacto-peptone, 1% dextrose, and 0.1% Bacto-casamino acids. Cultures in the late log phase of growth were centrifuged at 2,000 g for 10 minutes. The pellet was suspended in sterile water and adjusted to 0.3 absorbance at 600 nm (about 4×10^8 cells/ml).

Shoots were placed in large test tubes and submerged in a refrigerated glycol bath. Plants were held at test temperatures for 1 hr and then checked visually for ice nucleation. Freezing was evident as water-soaking and loss of turgor upon thawing.

Results and Discussion

Tomato plants were sprayed with about 0.5 ml/plant of 10^8 cells/ml of Erwinia herbicola M232A, a strain that is not active in ice nucleation. An INA strain (#26) was applied 24 hrs later and plants were freeze tested 24 hrs subsequent to inoculation with the INA strain. When the two strains were applied at the same concentration (10^8 cells/ml) freezing percentages were reduced 5-25% but the threshold temperature for ice nucleation was not reduced (Table 25). When the non-INA strain was applied at 100 and 1000 times the INA concentration, freezing percentages were reduced 15-20 and 15-35%, respectively (Table 26). Once again, the threshold freezing temperature was not reduced (with the possible exception of the hundredfold concentration difference). These data are not very promising in light of the fact that this is an idealized situation. The competitive bacteria (M232A) are introduced to "barren" plants and allowed to exploit this niche before introduction of the INA strain. Under natural conditions it is likely that the "competitive" (non-INA) bacteria would have to compete with previously established populations.

Tomato plants were sprayed with 10^8 cells/ml of P. syringae (INA) and/or Bacillus spp.-13 (non-INA) to determine whether freeze

Table 25. Percentage tomato plants frozen following inoculation with INA E. herbicola and/or non-INA (M232A) E. herbicola (20 replications per treatment were freeze tested)

Temperature (°C)	% Plants frozen					
	Control	M232A 10^8 H_2O	H_2O INA 10^7	M232A 10^8 INA 10^7	H_2O INA 10^8	M232A 10^8 INA 10^8
-3	0	0	5	0	0	0
-4	0	5	10	10	40	35
-5	0	5	70	30	90	65
-6	10	15	95	70	100	95
-7	25	25	100	90	100	95
-8	50	45	100	100	100	100

Table 26. Percentage tomato plants frozen following inoculation with INA E. herbicola and/or non-INA (M232A) E. herbicola (20 replications per treatment were tested)

Temperature (°C)	% Plants frozen					
	Control	M232A 10^8 H_2O	M232A 10^8 INA 10^5	H_2O INA 10^5	M232A 10^8 INA 10^6	H_2O INA 10^6
-3	0	0	0	0	0	0
-4	10	0	0	0	0	15
-5	25	10	20	55	60	80
-6	40	20	65	95	85	85
-7	60	65	85	100	95	100
-8	90	90	95	100	100	100

damage can be reduced when the INA population is introduced first. Freeze tests indicated that no protection was gained by this combination of bacteria (Table 27).

Tomato plants harboring INA bacteria (P. syringae) were treated with 100 ppm streptomycin and their freezing pattern was monitored over a time interval of about a week and a half. Streptomycin was either sprayed on the plant 24 hrs after bacterial inoculation or mixed with the bacterial suspension prior to inoculation. Throughout the experiment plants treated with the antibiotic-bacteria mixture froze very similar to control plants (Tables 28-31). Since the antibiotic was introduced to the bacterial suspension 2 hrs prior to inoculation it can be assumed that the cells came in contact with the antibiotic. This treatment entirely negated the ice-nucleating effects of the bacteria on the plants. In contrast, when the antibiotic was applied 24 hrs after bacterial inoculation results were very similar to plants treated only with bacteria. Apparently there was an interaction between the plant and antibiotic that blocked the antibiotic's action. The ice nucleation efficiency of the bacteria had declined after 264 hrs (11 days).

The experiment was repeated with very similar results (Tables 32-36). A treatment with the plants kept under a plastic cap (to maintain high relative humidity) and bacterial population counts were added. The percentage of plants frozen at warmer temperatures increased when the humidity was kept high while plants exposed to ambient conditions (60 ± 10% relative humidity) froze in decreasing numbers over

Table 27. Percentage tomato plants frozen following inoculation with P. syringae (INA) and/or Bacillus-13 (non-INA) (20 replications per treatment were tested)

Temperature (°C)	% Plants frozen			
	Control	H_2O Bacillus-13 10^8	P. syringae 10^8 Bacillus-13 10^8	P. syringae 10^8 H_2O
-3	0	0	0	0
-4	5	0	25	15
-5	20	15	90	100
-6	40	25	100	100
-7	55	65	100	100

Table 28. Percentages of tomato plants frozen 24 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by 100 ppm streptomycin 24 hrs later, and P. syringae

	24 hrs			
Temperature (°C)	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae
-3	0	0	0	0
-4	0	0	53	69
-5	0	0	100	100
-6	7	0	100	100
-7	33	47	100	100
-8	73	89	100	100

Table 29. Percentages of tomato plants frozen 48 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by 100 ppm streptomycin 24 hrs later, and P. syringae

	48 hrs			
Temperature (°C)	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae
-3	0	0	0	0
-4	0	0	87	100
-5	0	0	100	100
-6	0	0	100	100
-7	7	40	100	100
-8	40	87	100	100

Table 30. Percentages of tomato plants frozen 72 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by 100 ppm streptomycin 24 hrs later, and P. syringae

	72 hrs			
Temperature (°C)	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae
-3	0	0	0	0
-4	0	7	87	100
-5	0	13	100	100
-6	13	27	100	100
-7	40	87	100	100
-8	93	93	100	100

Table 31. Percentages of tomato plants frozen 264 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by 100 ppm streptomycin 24 hrs later, and P. syringae

Temperature (°C)	264 hrs			
	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae
-3	0	0	0	0
-4	0	0	13	13
-5	0	0	13	47
-6	0	0	27	73
-7	0	13	27	80
-8	53	47	60	100

Table 32. Percentages of tomato plants frozen 2 hrs after treatment with water (control) or P. syringae

Temperature (°C)	2 hrs	
	Control	P. syringae
-3	0	0
-4	0	40
-5	0	100
-6	60	100
-7	100	100

Table 33. Percentages of tomato plants frozen 24 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by streptomycin (100 ppm) 24 hrs later, and P. syringae at ambient and high humidity (cap)

Temperature (°C)	24 hrs				
	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae	P. syringae + cap
-3	0	0	50	0	20
-4	0	0	100	100	100
-5	0	0	100	100	100
-6	50	0	100	100	100
-7	100	60	100	100	100
-8	100	100	100	100	100

Table 34. Percentages of tomato plants frozen 72 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by streptomycin (100 ppm) 24 hrs later, and P. syringae at ambient and high humidity (cap)

	72 hrs				
Temperature (°C)	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae	P. syringae + cap
-3	0	0	0	10	40
-4	0	0	90	100	100
-5	0	0	100	100	100
-6	20	0	100	100	100
-7	40	0	100	100	100
-8	80	40	100	100	100
Population (cells/ g fresh wt)	0	0	1.5×10^4	2.4×10^5	2.6×10^5
Plant homogenate (°C)	-13.5	-13.8	-3.9	-3.0	-3.1

Table 35. Percentages of tomato plants frozen 120 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by streptomycin (100 ppm) 24 hrs later, and P. syringae at ambient and high humidity (cap)

	120 hrs				
Temperature (°C)	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae	P. syringae + cap
-3	0	0	10	10	30
-4	0	0	80	90	100
-5	0	0	90	90	100
-6	30	10	90	100	100
-7	60	10	90	100	100
-8	70	40	90	100	100
Population (cells/ g fresh wt)	0	0	1.4×10^4	1.2×10^4	8.0×10^5
Plant homogenate (°C)	-13.3	-11.7	-5.2	-5.3	-3.1

Table 36. Percentages of tomato plants frozen 240 hrs after treatment with water (control), a mixture of P. syringae and streptomycin (100 ppm), P. syringae followed by streptomycin (100 ppm) 24 hrs later, and P. syringae at ambient and high humidity (cap)

	240 hrs				
Temperature (°C)	Control	P. syringae + streptomycin 0 hrs	P. syringae + streptomycin 24 hrs	P. syringae	P. syringae + cap
-3	0	0	0	0	90
-4	0	0	0	20	100
-5	10	0	0	40	100
-6	10	20	10	60	100
-7	20	30	30	70	100
-8	50	60	80	90	100
Population (cells/ g fresh wt)	0	0	3.2×10^3	1.2×10^4	1.1×10^7
Plant homogenate (°C)	-10.6	-10.4	-10.0	-7.8	-2.5

240 hrs. The freezing behavior of plants was directly related to the bacterial populations and the mean freezing temperature of drops from plant homogenates. The bacterial population decreased in plants held at ambient humidity but increased in plants held at very high humidity. This finding should be taken into consideration when interpreting results from studies where plants were held at near 100% relative humidity (mist chamber) following inoculation (3,50,52).

Spectinomycin, another aminoglycoside, was evaluated for protection of tomato plants from bacterial ice nucleation. The results were more encouraging than those obtained with streptomycin (Table 37). The effects of P. syringae on plant freezing were virtually negated when 250 ppm of spectinomycin was added 24 hrs after bacterial inoculation. Reduction in freezing was observed with increased antibiotic concentration from 10 to 250 ppm. In order to substantiate the differing results from the two antibiotics, streptomycin and spectinomycin, were used in the same experiment (at the same concentration level). The results were the same as when used separately (Table 38). Spectinomycin effectively reduced the number of plants frozen while streptomycin did not.

Differences in effectiveness of the two antibiotics may be the results of the fate of the antibiotic in the plant and the location of the bacteria. If all of the bacteria remained on the plant surface it is likely that a topical spray would be effective. It was shown that streptomycin was effective when in contact with the bacterial cells. It is likely that a significant portion of the bacterial

Table 37. Mean percentage of tomato plants frozen following treatment with water (control), P. syringae (4×10^8 cells/ml), and spectinomycin (10-250 ppm)

Temperature (°C)	% Plants frozen					
	Control	P. syringae + spectinomycin 250 ppm	P. syringae + spectinomycin 100 ppm	P. syringae + spectinomycin 25 ppm	P. syringae + spectinomycin 10 ppm	P. syringae + H_2O
-3	5	0	0	0	15	10
-4	15	10	30	20	55	70
-5	15	20	45	50	70	100
-6	35	45	60	70	85	100
-7	60	70	85	90	95	100
-8	90	90	95	95	95	100

Table 38. Percentages of tomato plants frozen following treatment with water (control), P. syringae (4×10^8 cells/ml), streptomycin (100 ppm) and spectinomycin (100 ppm)

Temperature (°C)	% Plants frozen			
	Control	P. syringae + streptomycin	P. syringae + spectinomycin	P. syringae + H_2O
-3	0	5	0	45
-4	0	85	20	90
-5	5	100	55	95
-6	15	100	65	100
-7	40	100	75	100
-8	75	100	95	100

population is inside the leaf in the intercellular spaces. This habitat would provide more moisture than the external leaf surface. It is also probable that a portion of the population is superficial.

Poinsettia bracts were used to determine the importance of internal bacterial populations in ice nucleation. Bracts were chosen because they contain few or no stomates, a natural site of bacterial ingress. Bracts were sprayed, swabbed or injected with water or 10^6 cells/ml of P. syringae and freeze-tested 24 hrs later. The only treatment resulting in high-temperature ice nucleation was injection of the bacteria (Table 39). This was the only treatment with which it could be assured that bacteria were present intercellularly inside the plant organ. It is likely that bacterial populations were rapidly reduced to levels below the threshold necessary for ice nucleation by desiccation in the other treatments. Surface bacteria (living and/or dead) were not sufficient to bring about freezing of water inside the bracts.

Similar results were obtained with 'Calamondin' (Citrus madurensis L.) leaves (Table 40). The bacteria had to be injected into the leaves to observe high-temperature ice nucleation. Apparently the bacteria did not enter through stomates in numbers sufficient to cause ice nucleation. This may have been the result of stomatal closure due to the relatively low light intensity in the treatment room (100-200 $\mu E/m^2$ - s).

It is apparent from these data that bacteria inside the leaf can be responsible for ice nucleation. Therefore, an antibiotic must

Table 39. Number of poinsettia bracts frozen (10 replications per treatment were freeze tested 24 hrs after treatment)

Temperature (°C)	Number of poinsettia bracts frozen					
	H_2O			*P. syringae* 4×10^5 cells/ml		
	Spray	Swab	Inject	Spray	Swab	Inject
-3	0	0	0	0	0	0
-4	0	0	0	0	0	0
-5	0	0	0	0	0	8
-6	0	0	0	0	0	8
-7	0	0	0	0	0	8
-8	1	1	1	0	2	8
-9	4	3	5	3	4	10
-10	6	5	6	4	5	10

Table 40. Effect of method of inoculation of P. syringae on ice nucleation of 'Calamondin' leaves (10 replications per treatment)

Temperature (°C)	Freeze injury*					
	No nucleation	Spray H_2O	Inject H_2O	Spray P. syringae 4×10^7 cells/ml	Inject P. syringae 4×10^7 cells/ml	Nucleation with ice
-3	0	0	0	0	5	30
-4	0	0	0	0	17	30
-5	0	3	1	0	26	30
-6	0	3	4	0	30	30
-7	12	12	27	3	30	30
-8	24	30	30	27	30	30

*Injury rating based on water-soaking: 1 < 10%, 10% < 2 < 90%, 3 > 90% of leaf (multiplied by number of replications)

be effective against bacterial populations both on the surface and inside leaves. The cuticle of apple and coleus leaves did not stop penetration of streptomycin (26). However, it does not appear that the antibiotic remains in the intercellular spaces where the bacteria are located. It is rapidly taken up by the cells and accumulated in the vacuole by active processes [Pramer (1956) and Litmack and Pramer (1957), cited in Goodman (26)]. Citrus canker formation in citrus leaves was not stopped even when the antibiotic had been injection infiltrated (89). A degree of antibiotic effect was observed when cells were ruptured with carborundum. This evidence suggests that streptomycin is inactivated as a result of active uptake and accumulation by plant cells. It is presently not known whether or not spectinomycin is actively taken up and accumulated by plant cells. This does not seem likely in light of its effectiveness.

Streptomycin has two basic groups which are lacking in spectinomycin. This difference may play a role in differential effectiveness of the antibiotics. Streptomycin was placed in buffers (mono- and dibasic sodium phosphates) near physiological pH (6.2-7.7). Amino groups usually have pK values near 9.0 so the relative proportions of molecular species (conjugate acids and bases) would not be expected to differ greatly in this pH range. Streptomycin was effective in reducing bacterial ice nucleation in tomato plants when placed in buffer (Table 41). However, similar results were obtained at all pH values. Since the buffer solution is about 0.1 M, the effects of salt solutions on bacterial ice nucleation were examined. Streptomycin

Table 41. Effect of pH of antibiotic solution on bacterial ice nucleation of tomato plants. Streptomycin was applied at 100 ppm 24 hrs after bacterial inoculation

Temperature (°C)	% Tomato plants frozen				
	H_2O	*P. syringae* + streptomycin pH 7.7	*P. syringae* + streptomycin pH 6.7	*P. syringae* + streptomycin pH 6.2	*P. syringae* + H_2O
-3	0	13	27	7	100
-4	0	60	73	67	100
-5	7	93	87	93	100
-6	27	100	93	93	100
-7	67	100	100	100	100
-8	100	100	100	100	100

was placed in deionized water, buffer (pH 7.0), NaCl (0.1 M), and $CaCl_2$ (0.1 M). Salt solutions used were slightly less concentrated than physiological saline solution so the salts did not have a direct effect on the bacterial cells. Ice nucleation was not reduced when deionized water was the solvent but freezing percentages were substantially reduced when a salt solution (buffer, NaCl, $CaCl_2$) was employed (Table 42). It is possible that the dissolved salts interfere with the active uptake of the antibiotic by the plant cells.

Table 42. Effect of salts in antiobiotic solvent on reduction of bacterial ice nucleation of tomato plants (by P. syringae). Streptomycin was applied at 100 ppm 24 hrs after bacterial inoculation

Temperature (°C)	% Tomato plants frozen					
	H_2O	P. syringae + streptomycin DI H_2O	P. syringae + streptomycin Buffer	P. syringae + streptomycin NaCl	P. syringae + streptomycin $CaCl_2$	P. syringae + H_2O
-3	0	53	0	0	0	73
-4	13	100	33	47	27	93
-5	13	100	47	60	40	100
-6	40	100	53	60	40	100
-7	67	100	80	80	53	100
-8	73	100	93	93	73	100

CONCLUSIONS

Electrolyte leakage tests following controlled freezing are a better indicator of citrus cold hardiness than the leaf-freezing-point technique. The former test yields an indication of the tolerance of freezing while the latter indicates the degree of freeze avoidance. Leaves differing in hardiness exhibited very similar freezing behavior, indicating that differences in hardiness are the result of differences in the amount of frozen water tolerated. No significant differences were observed in liquid water content of unfrozen samples (g H_2O/g dry wt) or melting point depression in lemon, grapefruit, orange, and mandarin leaves. Tissues deviated from ideal freezing behavior in that less ice was formed than expected. Reduced ice formation could be explained by the formation of negative pressure potential during freezing. Negative pressure could arise due to the resistance to collapse of the cell wall during extracellular freezing. Treatments designed to reduce or eliminate negative pressure potential (tissue prefrozen in liquid nitrogen, expressed sap, and thawing curves) resulted in freezing behavior closer to ideal.

Tender plants are killed when frozen. Water-soaking and loss of turgor upon thawing are very good indicators of loss of viability. Plants inoculated with Pseudomonas syringae or Erwinia herbicola froze and were killed at higher temperatures than control plants without the

bacteria. Bacteria must be applied in concentrations of about 10^5 cells/ml or higher to observe high temperature ice nucleation. Ice nucleation efficiency decreased with time unless plants were held at very high relative humidity. Inoculation of an area as small as 4 mm with 4×10^8 cells/ml of P. syringae was sufficient to cause the entire leaf to freeze. Bacterial ice nucleation was not specific, causing all plants tested (tomato, soybean, pepper, begonia, marigold, coleus, calendula, and zinnia) to freeze at higher temperatures.

The ice nucleation efficiency of P. syringae is markedly affected by the culture and subsequent storage temperature. Bacteria grown at 22°C were much more efficient ice nucleators than bacteria grown at 30°C. Freezing efficiency could be increased by holding static cultures at 5°C. Decreasing efficiency of a culture held at a non-optimal temperature was the result of a reversible decrease in the concentration of nucleators rather than the progressive decline of individual ice nucleators. The viable bacterial concentration did not change during the course of this experiment, suggesting an on-off switching ability of the cells.

Spectinomycin, an aminoglycoside, reduced the percentage of (INA-bacteria-inoculated) tomato plants frozen. Similar results were obtained with streptomycin only if salts were added to the solvent. Competitive (non-nucleating) bacteria were not effective in reducing the number of plants frozen. Perhaps elucidation of the temperature-induced switching mechanism may prove to be a useful tool in control of bacterial ice nucleation.

APPENDICES

APPENDIX 1
VIABILITY TESTING

Leaf freezing points (actually the maxima of the exotherms) have been used to estimate the hardiness of citrus leaves (24,37). The validity of this method has come under question (112). In light of this, exotherm analyses were conducted with tangelo leaves to assess the feasibility of this test.

'Orlando' tangelo (Citrus reticulata Blanco X Citrus paradisi Macf.) leaves were randomly selected from a grove in Gainesville, Florida, for freezing point determinations. Leaves were placed in a domestic freezer at -15°C and exotherms were sensed by a thermister circuit. Exotherm analyses were carried out with tangelo leaves of various size (Figure 9). There was an apparent relationship between leaf size and supercooling. This was not unexpected because small sample size favors supercooling (94). This may be due to sequestering of the nuclei if the nuclei are uniformly distributed. The maximum temperature reached during the exotherm is a function of the freeze chamber temperature. Young reported that the leaf freezing temperature (maximum of the exotherm) was affected by the chamber temperature, hence the cooling rate (112). The freeze chamber temperature (-15°C) was sufficiently low to remove a portion of the heat of fusion before it was sensed. This was evident from the low values of the maxima of the exotherms (-5.9 to -8.6°C). These temperatures would require a

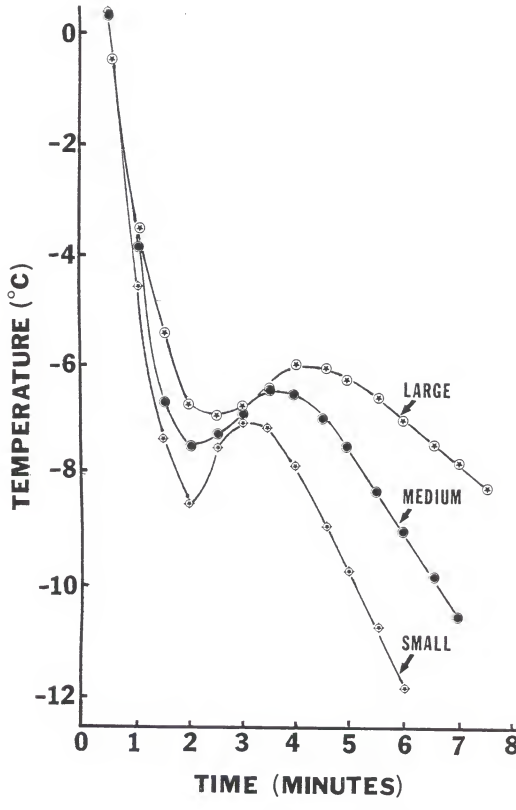


Figure 9. Cooling curves (with exotherms) of large, medium, and small 'Orlando' tangelo leaves.

melting point depression 3 to 4 times greater than observed in citrus leaves (29,30,31). Exotherm maxima would coincide with the killing temperature of a cold-acclimated leaf only under fortuitous circumstances (freezer temperature, hardening conditions, leaf size, and genotype).

Non-acclimated plant tissue is killed when frozen (6). Since supercooling is possible to -10°C (59) the ice nucleation temperature determines the killing point. This was readily apparent when non-acclimated 'Calamondin' (Citrus madurensis L.) leaves were nucleated with ice at 1°C intervals from -3 to -6°C (Table 43). The killing temperature (determined by electrolyte leakage) decreased about 1°C each time the nucleation temperature was decreased by the same amount. Water-soaking corresponded very closely with the killing temperature in these non-acclimated leaves. Leaves that were not nucleated froze and were killed at -7°C. These data indicate that the killing temperature of deacclimated plant tissue is a function of the ice nucleation temperature. This is a direct result of the fact that ice formation, not low temperature, is lethal. Leaf freezing points (not exotherm maxima) can be used to determine the killing temperature of deacclimated tissue if the cooling rate is naturally slow; of the order of one to several degrees (°C) per hour. A combination of factors will determine the ice nucleation temperature. These include the concentration and efficiency of heterogeneous nucleators (such as ice nucleation active bacteria) and the water status of the tissue (7,51,53). It did not appear that the citrus leaves used for freeze tests contained high-temperature nucleators (above -6°C).

Table 43. Lethal temperature of 'Calamondin' leaves ice nucleated at various temperatures. The temperature at which water-soaking was observed is indicated

Treatment	Lethal temperature (°C)	First appearance of water-soaking (°C)
Nucleated at -3°C	-3.5	-4
" " -4°C	-4.5	-4
" " -5°C	-5.5	-5
" " -6°C	-6.3	-6
Not nucleated	-7.1	-7

Electrolyte leakage viability tests (following controlled freezing) were adapted for citrus leaves to estimate cold hardiness. The leaves had to be sectioned because the waxy cuticle proved to be a barrier to diffusion (Table 44). Leaves that were frozen and killed did not lose all of the electrolytes unless the leaf was sectioned into strips about 1 cm in width. The conductivity of the leachate of killed leaves not sectioned was closer to values for leaves that were not frozen (controls). Killed leaves sectioned into 1 cm strips yielded conductivity values about the same as crushed leaves, a maximum value. Since slicing the leaves broke relatively few cells, this treatment did not introduce significant error (Table 45). Percent electrolyte leakage (initial reading/heat-killed reading x 100) was used to eliminate differences from leaf size variation.

Electrolyte leakage viability tests were used to monitor the cold hardiness of 'Hamlin' orange leaves on 'Sour' orange rootstock following controlled freezing. A typical hardiness evaluation is shown in Figure 10. The killing temperature on this date (1/5/81) was -7°C, the inflection point of the plot of percent conductivity vs. temperature. The standard deviation of the means (percent electrolyte leakage) was greatest at this value, being four times as large as at -3 to -5°C and from -9 to -10°C. This is probably due to the fact that a plant cell either is damaged or not injured as a result of exposure to a particular temperature. At temperatures warmer than the lethal temperature all values will be low, reflecting no injury (and a relatively small variation between replications). At temperatures much colder than the killing

Table 44. Conductivity of leachate 24 hrs after 'Hamlin' orange leaves were placed in deionized water at 26°C. Leaves were freeze killed and then sectioned (0.5-3.0 cm strips)

Electrolyte leakage	
Treatment	Conductivity (μS)
Control (not frozen)	10
Control	42
3.0 cm	160
1.0 cm	265
0.5 cm	265
Crushed	285
Crushed (not frozen)	290

Table 45. Percentage electrolyte leakage from 'Hamlin' orange leaves sectioned into strips (0.5-3.0 cm) or crushed with a mortar and pestle

Electrolyte leakage	
Treatment	% Electrolytes leached
Control	8.5
3.0 cm	6.8
1.0 cm	10.0
1.5 cm	10.5
Crushed	91.0

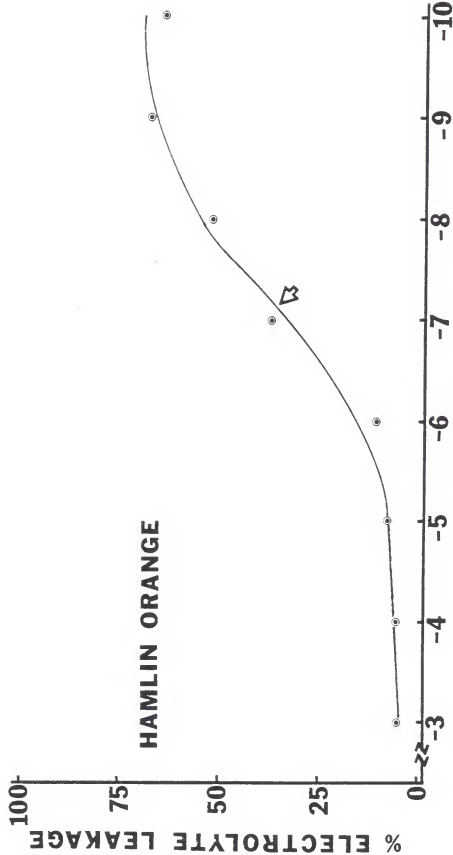


Figure 10. Electrolyte leakage viability test for 'Hamlin' orange leaves. The killing temperature is indicated (arrow).

point there will also be little variation since all cells have been killed. Near the killing temperature there will be both damaged and healthy cells due to slight differences in killing temperature. This gives rise to an injury range of about 2°C, with the killing temperature easily quantified by the inflection point of the plot of percent electrolyte leakage vs. temperature.

The leaves cold acclimated from the freezing point (-3°C) in October to -7 in January and deacclimated to -3°C by June (Figure 11). Water-soaking occurred at about the same temperature throughout the experiment (-3°C) indicating an acquisition of freeze tolerance. This acclimation-deacclimation sequence was the result of prevailing temperatures in Gainesville. Under these conditions 'Hamlin' orange leaves acquired 4°C of hardiness in about 4 months. Similar evaluations can be used as a local predictive tool for the need of frost protection measures.

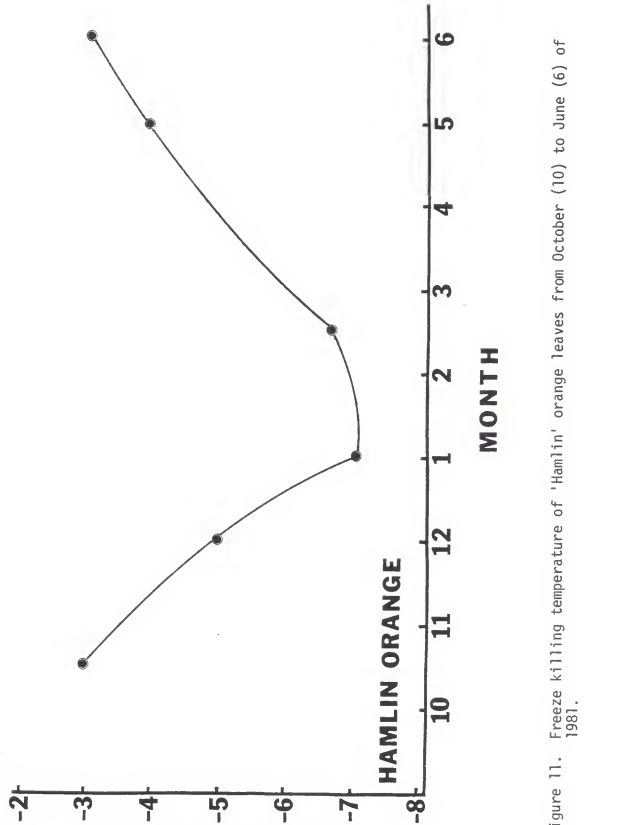


Figure 11. Freeze killing temperature of 'Hamlin' orange leaves from October (10) to June (6) of 1981.

APPENDIX 2
FIELD SURVEYS OF INA BACTERIA POPULATIONS

The role of ice nucleation active (INA) bacteria as causal agents of frost damage to plants is contingent upon several factors. The first prerequisite, ice nucleation of plant tissue at higher temperatures when the bacteria are present, has been established (3,51, 52). Furthermore, the bacteria not only must be ubiquitous but in concentrations high enough to cause tender plants to freeze. Pseudomonas syringae is considered to be widely distributed (14). Ice nucleation active bacteria, including P. syringae and Erwinia herbicola, were reported to be detected on 74 of 95 plant species surveyed in numbers high enough to account for ice nucleation (49). Finally, tender plants must have the capacity to supercool under field conditions in the absence of INA bacteria to make bacterial ice nucleation more than an academic curiosity. These conditions have not been explored sufficiently to make generalizations regarding the role of INA bacteria in frost damage to tender plants in the field.

The objective of this research was to make a preliminary field survey of the INA bacteria populations in the Gainesville, Florida, area.

Plant samples were weighed before crushing in sterile deionized water. Suspensions were either frozen directly (20 µl drops) or dilution plated and colonies of each morphology and color were tested for ice nucleation activity on a Peltier freeze plate.

Dew was collected from various plant leaves with a syringe on the morning of October 6, 1980. Bacterial populations ranged from 6.4×10^4 cells/ml dew (photinia) to 9.4×10^5 for pecan (Table 46). Virtually no fluorescent colonies were detected on the KMB agar indicating that if INA bacteria are present they are most likely E. herbicola. On January 14, 1981, two dew samples were collected from clover and frozen along with INA and water controls (Table 47). No high-temperature ice nucleators were present in the dew samples. This suggests that, in some cases, dew is void of nucleators and is probably frozen by nucleators in the plant tissue.

Peach flowers, flower buds, and weed leaves were collected from a peach orchard on February 10, 1981. Plant samples were crushed in sterile deionized water and frozen. The mean freezing temperatures indicate that ice nucleators were present on the unidentified weed leaves (Table 48). A bacterium isolated fom the leaves proved to be a fluorescent pseudomonad. This isolate was cultured in CPG medium and sprayed on pepper plants along with the standard isolate of P. syringae (C-9). More efficient freezing was observed with the standard isolate (Table 49). The surviving population was found to be slightly higher on plants inoculated with the standard isolate. Therefore, in order to determine if the lower percentage of plants frozen was due to a lesser concentration of bacteria, drops of both isolates [C-9 (standard) and W-1 (from weed)] were freeze tested. The mean freezing temperature of 20 µl drops indicate that isolate W-1 is intrinsically a slightly poorer ice nucleator than isolate C-9 (Table 50).

Table 46. Bacterial populations in dew collected from plant leaves

Plant sample	Bacterial population[z]
Pecan	9.4×10^5
Rose	2.1×10^5
Citrus	3.6×10^5
Avocado	2.1×10^5
Fig	2.1×10^5
Grass	2.5×10^5
Photinia	6.4×10^4

[z]Cells/ml dew

Table 47. Mean freezing temperature for two dew samples from clover and *P. syringae* and water controls

Plant sample	Mean freezing temperature (°C)
P. syringae	-4.6
Clover dew	-17.7
Clover dew	-15.2
Water	-17.7

Table 48. Freezing temperatures of plant homogenates. Tissues were crushed in 5 ml of sterile water and 20 µl drops were frozen

Plant sample	Freezing temperature (°C)
Peach flower	-8.5
Peach flower bud	-13.0
Peach leaf bud	-9.2
Weed leaf	-4.7
Water	-15.6

Table 49. Percentages of pepper shoots frozen following inoculation with P. syringae isolate C-9 or W-1 or with water. Freeze test and bacterial population determination carried out 48 hrs after treatment

	% Pepper plants frozen		
Temperature (°C)	Control	P. syringae C-9	P. syringae W-1
-3	0	3	0
-4	3	93	50
-5	13	100	100
-6	28	100	100
-7	43	100	100
-8	60	100	100
Bacterial population (cells/g fresh wt)	<100	2.2×10^5	8.3×10^4

Table 50. Mean freezing temperatures (°C) of P. syringae isolates C-9 and W-1

Concentration (cells/ml)	Freezing temperature (°C)	
	P. syringae C-9	P. syringae W-1
4×10^8	-3.1	-4.3
4×10^7	-8.3	-8.7

Peach leaves and flowers were collected on March 1, 1981, from another orchard in Gainesville (about 10 years old). Once again the samples did not contain high temperature ice nucleators in spite of bacterial populations of 1×10^4 (leaves) and 5×10^5 (flowers) cells/g fresh wt (Figure 12).

Leaves from coleus (Coleus blumei) and dusty miller (Centaurea cineraria) plants were crushed in sterile deionized water in June of 1981. The freezing temperatures of the plant suspensions indicate the presence of ice nucleators (Figure 13). Dusty miller plants which were exposed to direct sun resulted in 50% freezing at -7.2°C while shaded coleus plants yielded drops which were 50% frozen at -5.7°C. Leaves from citrus and blueberry plants were collected because plants predominantly exposed to sunny and shady conditions were available. In all cases the bacterial populations from leaves in the sun were smaller than shaded populations (Table 51). The difference ranged from 2.5 to 10 times greater for shaded leaves. Total populations ranged from 8.0×10^2 to 6.1×10^3 for leaves in the sun and from 8.0×10^3 to 2.0×10^4 for leaves in a shady location.

Bacterial populations exposed to UV-β radiation have been observed to decrease rapidly in number (25,74,75). These studies demonstrated the bactericidal nature of UV-β radiation on airborne bacteria and in-vitro cultures. Tomato plants were placed under various dosage levels of UV-β radiation to determine the effect on epiphytic populations of P. syringae.

Although ice nucleation was not affected, bacterial populations decreased with increasing UV dosage level (Table 52). It is possible

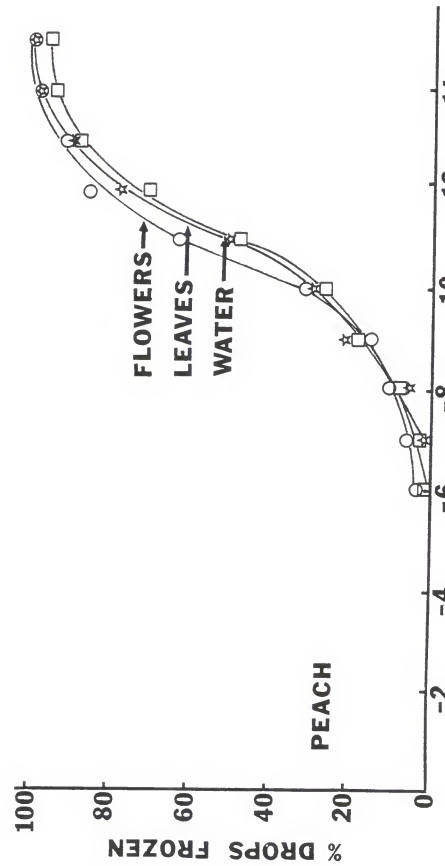


Figure 12. Freezing temperatures of 20 µl drops from tissue homogenates.

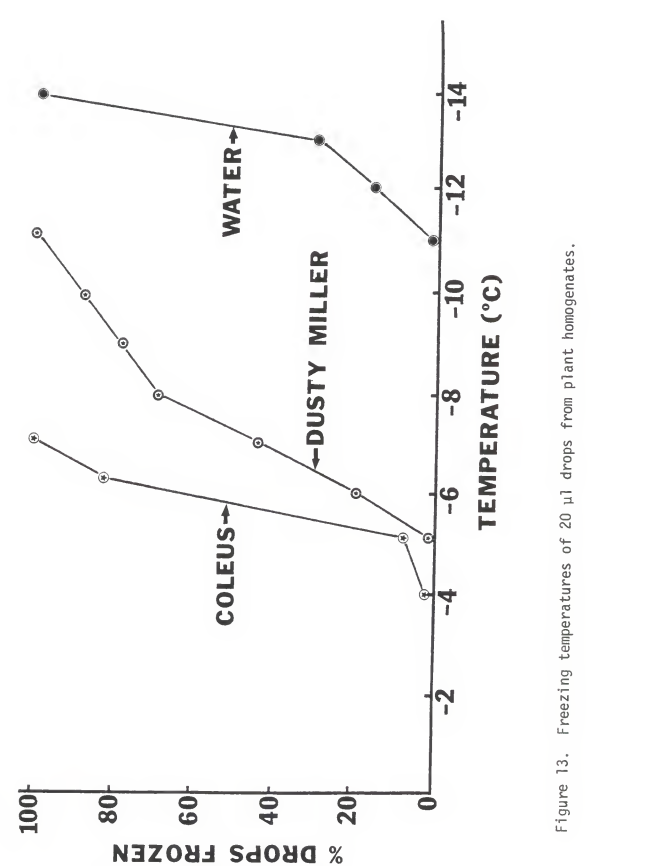


Figure 13. Freezing temperatures of 20 µl drops from plant homogenates.

Table 51. Bacteria population from leaves in sunny and shady locations

Plant	Bacteria population	
	Location	Population[z]
Orange	Sun	3.7×10^3
Orange	Shade	2.0×10^4
Blueberry	Sun	8.0×10^2
Blueberry	Shade	8.0×10^3
Tangelo	Sun	6.1×10^3
Tangelo	Shade	1.5×10^4

[z]Cells/g fresh wt

Table 52. Effects of ultraviolet-β radiation on bacterial ice nucleation and bacterial population

Temperature (°C)	% Tomato plants frozen				
		P. syringae 4 x 10^8 cells/ml			
	Control	Control	Baseline	20%	50%
-3	0	27	13	0	20
-4	0	67	93	67	60
-5	7	93	93	100	93
-6	20	93	100	100	100
-7	47	93	100	100	100
-8	87	100	100	100	100
Total dose (J/m^2)	20	20	1486	1817	2160
Population (cells/g fresh wt)	0	1.3 x 5^5	2.0 x 10^4	1.3 x 10^4	3.0 x 10^3

that ice nucleation temperatures may be reduced with an inoculum concentration lower than 4×10^8 cells/ml. The baseline dosage (1486 J/m^2) is comparable with solar levels in Gainesville, Florida.

Data indicate that the previous report on INA bacteria distribution (49) may be somewhat optimistic for Florida conditions. It appears likely that solar UV-β radiation plays a role in limiting epiphytic bacterial populations.

LITERATURE CITED

1. Alden, J., and R. K. Hermann. 1971. Aspects of the cold hardiness mechanism in plants. Bot. Rev. 37:37-142.

2. Anderson, P., J. Davies, and B. D. Davis. 1967. Effect of spectinomycin on polypeptide synthesis in extracts of Escherichia coli. J. Mol. Biol. 29:203-215.

3. Arny, D. C., S. E. Lindow, and C. D. Upper. 1976. Frost sensitivity of Zea mays increased by application of Pseudomonas syringae. Nature 262:282-284.

4. Barrett, C. R., W. D. Nix, and A. S. Tetelman. 1973. p. 161-170 In The principles of engineering materials. Prentice-Hall, Englewood Cliffs, New Jersey.

5. Burke, M. J., M. F. George, and R. G. Bryant. 1975. Water in plant tissues and frost hardiness. p. 111-135 In Water relations of foods. R. B. Duckworth (ed.). Academic Press, New York.

6. Burke, M. J., L. V. Gusta, H. A. Quamme, C. J. Weiser, and P. H. Li. 1976. Freezing and injury in plants. Ann. Rev. Plant Physiol. 27:507-528.

7. Cary, J. W., and H. F. Mayland. 1970. Factors influencing freezing of supercooled water in tender plants. Agron. J. 62: 715-719.

8. Chandler, W. H. 1954. Cold resistance in horticultural plants: a review. Proc. Amer. Soc. Hort. Sci. 64:552-572.

9. Chen, P. M., M. J. Burke, and P. H. Li. 1976. The frost hardiness of several solanum species in relation to the freezing of water, melting point depression, and tissue water content. Bot. Gaz. 137:313-317.

10. Chen, P. M., and P. H. Li. 1977. Induction of frost hardiness in stem cortical tissues of Cornus stolonifera Michx. by water stress. II. Biochemical changes. Plant. Physiol. 59:240-243.

11. Chen, P. M., P. H. Li, and M. J. Burke. 1977. Induction of frost hardiness in stem cortical tissues of Cornus stolonifera Michx. by water stress. I. Unfrozen water in cortical tissues and water status in plants and soil. Plant Physiol. 59:236-239.

12. Chen, P. M., P. H. Li, and C. J. Weiser. 1975. Induction of frost hardiness in red-osier dogwood stems by water stress. HortScience 10:372-374.

13. Cooper, W. C., and B. S. Gorton. 1954. Freezing tests with small trees and detached leaves of grapefruit. Proc. Amer. Soc. Hort. Sci. 63:167-172.

14. Crosse, J. E., and C. M. E. Garrett. 1963. Studies on the bacteriophagy of Pseudomonas mors-prunorum, P. syringae, and related organisms. J. Appl. Bact. 26:159-177.

15. Davies, J., P. Anderson, and B. D. Davis. 1965. Inhibition of protein synthesis by spectinomycin. Science 149:1096-1098.

16. Fennema, O. R., W. D. Powrie, and E. H. Marth. 1973. Low temperature preservation of foods and living matter. Marcel Dekker, New York.

17. Fowler, D. B., L. V. Gusta, and N. J. Tyler. 1981. Selection for winter hardiness in wheat. III. Screening methods. Crop Science 21:896-901.

18. Fuchigami, L. H., C. J. Weiser, and D. R. Evert. 1971. Induction of cold acclimation in Cornus stolonifera Michx. Plant Physiol. 47:98-103.

19. Fuchigami, L. H., C. J. Weiser, and D. R. Evert. 1971. A translocatable cold hardiness promoter. Plant Physiol. 47:164-167.

20. Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring. 1972. The molecular basis of antibiotic action. J. Wiley & Sons, New York.

21. Gardner, F. E., and G. E. Horanic. 1963. Influence of various rootstocks on the cold resistance of the scion variety. Proc. Fla. St. Hort. Soc. 76:105-110.

22. George, M. F., M. J. Burke, H. M. Pellett, and A. G. Johnson. 1974. Low temperature exotherms and woody plant distribution. HortScience 9:519-522.

23. George, M. F., M. J. Burke, and C. J. Weiser. 1974. Supercooling in overwintering azalea flower buds. Plant Physiol. 54:29-35.

24. Gerber, J. F., and F. Hashemi. 1965. The freezing point of citrus leaves. Proc. Amer. Soc. Hort. Sci. 86:220-225.

25. Gibbins, L. N., and D. L. Peterson. 1978. Responses of Erwinia herbicola Y46 and non-pigmented mutant to ultraviolet radiation and visible light; survival curves and photo-reactivation. p. 443-450. Proc. 4th Int. Conf. Plant Path. Bact. Angers.

26. Goodman, R. N. 1962. Systemic effects of antibiotics. p. 165-181. In M. Woodbine (ed.), Antibiotics in agriculture. Butterworths, London.

27. Green, D. G., and C. D. Ratzlaff. 1975. An apparent relationship of soluble sugars with hardiness in winter wheat varieties. Can. J. Bot. 53:2198-2201.

28. Gusta, L. V., M. J. Burke, and A. C. Kapoor. 1975. Determination of unfrozen water in winter cereals at subfreezing temperatures. Plant Physiol. 56:707-709.

29. Haas, A. R. C., and F. F. Halma. 1928. Physical and chemical characteristics of expressed citrus leaf sap and their significance. Bot. Gaz. 85:457-461.

30. Haas, A. R. C., and F. F. Halma. 1931. Sap concentration and inorganic constituents of mature citrus leaves. Hilgardia 5:407-424.

31. Halma, F. F., and A. R. C. Haas. 1928. Effect of sunlight on sap concentration of citrus leaves. Bot. Gaz. 86:102-106.

32. Hearn, C. J., W. C. Cooper, R. O. Register, and R. Young. 1964. Influence of variety and rootstock upon freeze injury to the citrus trees in the 1962 Florida freeze. The Citrus Industry 3: 17-21.

33. Hendershott, C. H. 1962. The responses of orange trees and fruits to freezing temperatures. Proc. Amer. Soc. Hort. Sci. 80:247-254.

34. Hirano, S. S., E. A. Maher, A. Kelman, and C. D. Upper. 1978. Ice nucleation activity of fluorescent plant pathogenic pseudomonads. Proc. 4th Int. Conf. Plant Path. Bact. Angers. 717-724.

35. Howell, G. W., and C. J. Weiser. 1970. Similarities between the control of flower initiation and cold acclimation in plants. HortScience 5:18-20.

36. Hurst, C., T. C. Hall, and C. J. Weiser. 1967. Reception of the light stimulus for cold acclimation in Cornus stolonifera Michx. HortScience 2:164-166.

37. Hutcheson, C. E., and W. J. Wiltbank. 1972. The freezing point of detached leaves as a measure of cold hardiness of young budded citrus plants. HortScience 7:27-28.

38. Irving, R. M., and F. O. Lanphear. 1967. The long day leaf as a source of cold hardiness inhibiters. Plant Physiol. 42:1384-1388.

39. Jackson, L. K., and J. F. Gerber. 1963. Cold tolerance and freezing point of citrus seedlings. Proc. Fla. St. Hort. Soc. 76:70-74.

40. Jones, W. W., and M. L. Steinachen. 1951. Seasonal changes in concentration of sugar and starch in leaves and twigs of citrus trees. Proc. Amer. Soc. Hort. Sci. 58:1-4.

41. Kaku, S. 1973. High ice nucleating ability in plant leaves. Plant & Cell Physiol. 14:1035-1038.

42. Kassander, A. R., L. L. Sims, and J. E. McDonald. 1955. Observations of freezing nuclei over the southwestern U.S. p. 392-403. In Artificial stimulation of rain. Pergamon Press, New York.

43. Knothe, H., V. Krcmery, and B. Wiedemann. 1974. A new type of r plasmid in Serratia marcescens transferring gentamicin, tobramycin, dkb, lividomycin, and kanamycin resistance. p. 217-222. In S. Mituhashi and H. Hashimoto (eds.), Microbial drug resistance. University Park Press, Tokyo.

44. Kontomichalou, P., and E. Papachistou. 1974. Multiresistance plasmids in Pseudomonas aeruginosa highly resistant to gentamicin. p. 329-348. In S. Mituhashi and H. Hashimoto (eds.), Microbial drug resistance. University Park Press, Tokyo.

45. Larsen, R. P. 1970. Introduction to the symposium. HortScience 5:402.

46. Leben, C., and T. D. Miller. 1973. A pathogenic pseudomonad from healthy field-grown soybean plants. Phytopath. 63:1464-1467.

47. Levitt, J. 1980. Responses of plants to environmental stresses. p. 166-168. Vol. 1. Academic Press, New York.

48. Li, P. H., C. J. Weiser, and R. van Huystee. 1966. The relation of cold resistance to the status of phosphorus and certain metabolites in red-osier dogwood (Cornus stolonifera Michx.). Plant & Cell Physiol. 7:475-484.

49. Lindow, S. E., D. C. Arny, and C. D. Upper. 1978. Distribution of ice nucleation-active bacteria on plants in nature. Appl. & Environ. Microbiol. 36:831-838.

50. Lindow, S. E., D. C. Arny, and C. D. Upper. 1978. Erwinia herbicola: a bacterial ice nucleus active in increasing frost injury to corn. Phytopath. 68:523-527.

51. Lindow, S. E., D. C. Arny, and C. D. Upper. 1982. Bacterial ice nucleation: a factor in frost injury to plants. Plant physiol. 70:1084-1089.

52. Lindow, S. E., D. C. Arny, C. D. Upper, and W. R. Barchet. 1978. The role of bacterial ice nuclei in frost injury to sensitive plants. p. 249-263. In P. H. Li and H. Sakai (eds.). Plant cold hardiness and freezing stress. Academic Press, New York.

53. Lindow, S. E., S. S. Hirano, W. R. Barchet, D. C. Arny, and C. D. Upper. 1982. Relationship between ice nucleation frequency of bacteria and frost injury. Plant Physiol. 70:1090-1093.

54. Lowbury, E. J. L., and G. A. J. Aryliffe. 1974. Drug resistance in antimicrobial therapy. p. 4. C. C. Thomas, Springfield, Illinois.

55. Lucas, J. W. 1954. Subcooling and ice nucleation in lemons. Plant Physiol. 29:245-251.

56. Maki, L. R., E. L. Galyan, M. Chang-chien, and D. R. Caldwell. 1974. Ice nucleation induced by Pseudomonas syringae. Appl. Microbiol. 28:456-459.

57. Maki, L. R., and D. M. Garvey. 1975. Bacterially induced ice nucleation. Trans. Am. Geophys. Union. 56:994 (Abstr.)

58. Marcellos, H., and M. J. Burke. 1979. Frost injury in wheat: ice formation and injury in leaves. Aust. J. Plant Physiol. 6:513-521.

59. Marcellos, H., and W. V. Single. 1976. Ice nucleation on wheat. Agr. Meteorol. 16:125-129.

60. Marcellos, H., and W. V. Single. 1979. Supercooling and heterogeneous nucleation of freezing in tissues of tender plants. Cryobiol. 16:74-77.

61. Mayland, H. F., and J. W. Cary. 1970. Frost and chilling injury to growing plants. Adv. Agron. 22:203-234.

62. McKenzie, J. S., C. J. Weiser, and M. J. Burke. 1974. Changes in water relations of Cornus stolonifera during cold acclimation. J. Amer. Soc. Hort. Sci. 99:223-228.

63. McKenzie, J. S., C. J. Weiser, and M. J. Burke. 1974. Effects of red and far red light on the initiation of cold acclimation in Cornus stolonifera Michx. Plant Physiol. 53:783-789.

64. Okabe, N., and M. Goto. 1963. Bacteriophages of plant pathogens. Ann. Rev. Phytopathol. 1:397-418.

65. Otaya, H., S. Machihara, M. Yoshimuro, and M. Yamamoto. 1974. Multiply drug-resistant Escherichia coli isolated from clinical specimens. p. 201-208. In S. Mituhashi, and H. Hashimoto (eds.). Microbial drug resistance. University Park Press, Tokyo.

66. Palta, J. P., J. Levitt, E. J. Stadelman, and M. J. Burke. 1977. Dehydration of onion cells: a comparison of freezing vs. desiccation and living vs. dead cells. Physiol. Plant. 41:273-279.

67. Parker, J. 1960. Survival of woody plants at extremely low temperatures. Nature 187:1133.

68. Parsons, L. R. 1978. Water relations, stomatal behavior, and root conductivity of red-osier dogwood during acclimation to freezing temperatures. Plant Physiol. 62:64-70.

69. Paulin, J.-P., and J. Luisetti. 1978. Ice nucleation activity among phytopathogenic bacteria. Proc. 4th Int. Conf. Plant. Path. Bact. Angers. 725-731.

70. Proebsting, E. 1963. The role of air temperatures and bud development in determining hardiness of dormant Elberta peach fruit buds. Proc. Amer. Soc. Hort. Sci. 83:259-269.

71. Proebsting, E. L., Jr., and A. Sakai. 1979. Determining T50 of peach flower buds with exotherm analysis. HortScience 14:597-598.

72. Rajashekar, C., and M. J. Burke. 1978. The occurrence of deep undercooling in the genera Pyrus, Prunus, and Rosa: a preliminary report. p. 213-225. In Plant cold hardiness and freezing stress. P. H. Li and A. Sakai (eds.). Academic Press, New York.

73. Rasmussen, D. H., and A. P. MacKenzie. 1972. p. 126-145. In Water structure at the water-polymer interface. Plenum Pub., New York.

74. Rentschler, H. C., and R. Hagy. 1942. Bactericidal action of ultraviolet radiation on air-borne organisms. J. Bact. 44:85-94.

75. Riley, R. L., and J. E. Kaufman. 1972. Effect of relative humidity on the inactivation of airborne Serratia marcescens by ultraviolet radiation. Appl. Microbiol. 23:1113-1120.

76. Sakai, A. 1960. Survival of the twig of woody plants at -196°C. Nature 185:393-394.

77. Sakai, A. 1966. Studies of frost hardiness in woody plants. II. Effects of temperature on hardening. Plant Physiol. 41:353-359.

78. Sakai, A. 1966. Survival of plant tissue at super-low temperatures. IV. Cell survival with rapid cooling and rewarming. Plant Physiol. 41:1050-1054.

79. Sakai, A. 1979. Deep supercooling of winter flower buds of Cornus florida L. HortScience 14:69-70.

80. Schneider, R. W., and R. G. Grogan. 1977. Bacterial speck of tomato: sources of inoculum and establishment of a resident population. Phytopath. 67:388-394.

81. Schneider, R. W., and R. G. Grogan. 1977. Tomato leaf trichomes, a habitat for resident populations of Pseudomonas tomato. Phytopath. 67:898-902.

82. Schnell, R. C., and G. Vali. 1972. Atmospheric ice nuclei from decomposing vegetation. Nature 236:163-165.

83. Sharples, G. C., and L. Burkhart. 1954. Seasonal changes in carbohydrates in the Marsh grapefruit tree in Arizona. Proc. Amer. Soc. Hort. Sci. 63:74-80.

84. Siminovitch, D., F. G. Gfeller, and B. Rheame. 1967. The multiple character of the biochemical mechanism of freezing resistance of plant cells. p. 93-117. In Cellular injury and resistance in freezing organisms. E. Asahina (ed.). Inst. Low Temp. Sci., Hokkaido Univ., Sapporo, Japan.

85. Single, W. V., and H. Marcellos. 1974. Studies on frost injury to wheat. IV. Freezing of ears after emergence from the leaf sheath. Aust. J. Agric. Res. 25:679-686.

86. Smith, H. W. 1962. The effects of the use of antibiotics on the emergence of antibiotic-resistant disease-producing organisms in animals. p. 374-385. In M. Woodbine (ed.). Antibiotics in agriculture. Butterworth, London.

87. Sparling, P. F. 1968. Kasugamycin resistance: 30s ribosomal mutation with an unusual location on the Escherichia coli chromosome. Science 167:56-58.

88. Spiers, J. M., and A. D. Draper. 1974. Effect of chilling on bud break in rabbiteye blueberry. J. Amer. Soc. Hort. Sci. 99:398-399.

89. Stall, R. E. Personal communication.

90. Stout, D. G. 1981. Dehydration strain avoidance and tolerance in plant cold hardiness. J. Theor. Biol. 88:513-521.

91. Sukumaran, N. P., and C. J. Weiser. 1972. An excised leaflet test for evaluating potato frost tolerance. HortScience 7:467-468.

92. Sukumaran, N. P., and C. J. Weiser. 1972. Freezing injury in potato leaves. Plant Physiol. 50:564-567.

93. van Huystee, R., C. J. Weiser, and P. Li. 1967. A chronology of cold acclimation and phenological characters in red-osier dogwood under natural and controlled photoperiod and temperature. Bot. Gaz. 128:200-205.

94. Vali, G. 1971. Quantitative evaluation of experimental results on the heterogeneous freezing nucleation of supercooled liquids. J. Atmos. Sci. 28:402-409.

95. Vali, G., M. Christensen, R. W. Fresh, E. L. Galyan, L. R. Maki, and R. C. Schnell. 1976. Biogenic ice nuclei. Part II. Bacterial sources. J. Atmos. Sci. 33:1565.

96. Vali, G., and E. J. Stansbury. 1966. Time-dependent characteristics of the heterogeneous nucleation of ice. Can. J. Physics 44:477-502.

97. Weiser, C. J. 1971. Cold resistance and acclimation in woody plants. HortScience 5:403-410.

98. Weiser, C. J., H. A. Quamme, E. L. Proebsting, J. M. Burke, and G. Yelenosky. 1979. Plant freezing injury and resistance. p. 55-84. In B. J. Barfield and J. F. Gerber (eds.). Modification of the aerial environment of plants. Amer. Soc. Agric. Eng., St. Joseph, Michigan.

99. Wood, G. R., and A. G. Walton. 1979. Homogeneous nucleation kinetics of ice from water. J. Appl. Physics 41:3027-3036.

100. Yelenosky, G. 1971. Effect of light on cold-hardening of citrus seedlings. HortScience 6:234-235.

101. Yelenosky, G. 1975. Cold hardening in citrus stems. Plant Physiol. 56:540-543.

102. Yelenosky, G. 1976. Ice tolerance of cold-hardened 'Valencia' orange wood. Cryobiol. 13:243-247.

103. Yelenosky, G. 1978. Cold hardening 'Valencia' orange trees to tolerate -6.7°C without injury. J. Amer. Soc. Hort. Sci. 103:449-452.

104. Yelenosky, G. 1978. The effect of withholding water on cold hardiness of 'Valencia' orange and 'Star Ruby' grapefruit trees in controlled freezes. Proc. Fla. St. Hort. Soc. 91:18-20.

105. Yelenosky, G. 1979. Accumulation of free proline in citrus leaves during cold hardening of young trees in controlled temperature regimes. Plant Physiol. 64:425-427.

106. Yelenosky, G. 1979. Water-stress-induced cold hardening of young citrus trees. J. Amer. Soc. Hort. Sci. 104:270-273.

107. Yelenosky, G., and C. L. Guy. 1977. Carbohydrate accumulation in leaves and stems of 'Valencia' orange at progressively colder temperatures. Bot. Gaz. 138:13-17.

108. Yelenosky, G., and C. J. Hearn. 1967. Cold damage to young mandarin-hybrid trees on different rootstocks in flatwood soil. Proc. Fla. St. Hort. Soc. 80:53-56.

109. Yelenosky, G., and R. Young. 1977. Cold hardiness of orange and grapefruit trees on different rootstocks during the 1977 freeze. Proc. Fla. St. Hort. Soc. 90:49-53.

110. Yelenosky, G., R. Young, C. J. Hearn, H. C. Barrett, and D. J. Hutchison. 1981. Cold hardiness of citrus trees during the 1981 freeze in Florida. Proc. Fla. St. Hort. Soc. 94:46-51.

111. Young, R. 1963. Climate-cold hardiness—citrus. J. Rio Grande Valley Hort. Soc. 17:3-14.

112. Young, R. H. 1966. Freezing points and lethal temperatures of citrus leaves. Proc. Amer. Soc. Hort. Sci. 88:272-279.

113. Young, R. 1969. Cold hardening citrus seedlings as related to artificial hardening conditions. J. Amer. Soc. Hort. Sci. 94: 612-614.

114. Young, R. 1969. Cold hardening in 'Redblush' grapefruit as related to sugars and water soluble proteins. J. Amer. Soc. Hort. Sci. 34:252-254.

115. Young, R. 1970. Induction of dormancy and cold hardiness in citrus. HortScience 5:411-413.

116. Young, R., and W. D. Bell. 1974. Photosynthesis in detached leaves of cold-hardened citrus seedlings. J. Amer. Soc. Hort. Sci. 99:400-403.

117. Young, R., and E. O. Olson. 1963. Freeze injury to citrus trees on various rootstocks in the lower Rio Grande Valley of Texas. Proc. Amer. Soc. Hort. Sci. 83:337-343.

118. Young, R. H., and A. Peynado. 1962. Growth and cold hardiness of citrus and related species when exposed to different night temperatures. Proc. Amer. Soc. Hort. Sci. 81:238-243.

119. Young, R., and A. Peynado. 1965. Changes in cold hardiness and certain physiological factors of 'Red Blush' grapefruit seedlings as affected by exposure to artificial hardening temperatures. Proc. Amer. Soc. Hort. Sci. 86:244-252.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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